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On October 23, 2001

TOWNSEND and TOWNSEND and CREW LLP

By: Debra A DeBello
Debra Ann DeBello

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Paulson *et al.* Application No.: 09/007,741 Filed: January 15, 1998 For: PRACTICAL *IN VITRO* SIALYLATION OF RECOMBINANT GLYCOPROTEINS	Examiner: Manjunath Rao Art Unit: 1652 DECLARATION OF DAVID A. ZOPF, M.D.

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, David A. Zopf, M.D. declare as follows:

1. I am Vice President of New Product Development of Neose Technologies, Inc. (hereinafter "Neose"). Prior to joining Neose, I was Vice President and Chief Operating Officer of BioCarb, Inc. In addition, I am an editorial board member of *Archives of Biochemistry and Biophysics*. My *Curriculum Vitae* is attached.

2. I am submitting this declaration to address whether, from an objective point of view, the subject matter of the present application was "obvious" to those working in the art at the time the invention was made.

3. Attached herewith as Exhibit 1, is a copy of a letter from Professor Dr. James E. Bailey, Institute of Biotechnology, ETH-Zürich, CH-8093 Zurich, Switzerland. I believe that the attached letter is evidence of skepticism by an Expert, which constitutes strong evidence of nonobviousness of the claimed invention.

4. In general, the letter expresses disbelief that an *in vitro* glycosylation process is commercially viable. Dr. Bailey states therein that "the process complications and costs associated with producing and utilizing a glycosyltransferase and donor substrate make the exogenous manipulation of glycosylation far less attractive than engineering the cells to maximize the production of the desired glycoform." Dr. Bailey continues by saying, "[f]ailure of much simpler cofactor-requiring enzyme catalyzed reactions to gain industrial success in competition with whole-cell biocatalysts speaks very strongly in my opinion *against* the competitive prospects of *in vitro* remodeling of glycosylation." [Emphasis added].

5. In addition to the foregoing evidence of skepticism by an Expert, the technology as presently claimed has enjoyed commercial success. It is my understanding, that this too is objective evidence of nonobviousness.

6. In my capacity as Vice President of New Product Development of Neose, I have directly participated in negotiating agreements with more than 20 companies to assess the feasibility of the technology for *in vitro* sialylation of recombinant therapeutic glycoproteins in development. All feasibility studies completed to date have been successful. Many of these successful feasibility studies have led to ongoing negotiations for commercial licenses to the technology for large-scale manufacture of

human glycoprotein therapeutics. In addition, the present technology is being employed as an essential part of ongoing collaborative research and development agreements with other companies to develop commercial manufacturing methods for cancer vaccines and treatments for neurological diseases.

7. Attached is Exhibit 2 which sets forth consistent improvement in glycosylation when various companies used the claimed invention. As shown therein, the percentage of sites wherein sialic acid could potentially be present, but was missing because the cell that had expressed the glycoprotein failed to add sialic acid as a terminal sugar, ranged from, 15% to 85%. After using the claimed invention, sialic acid occupied greater than 90% of possible sites.

8. In another example, a particular company under a use agreement, published the results. The results are set forth in paragraphs 9 and 10. This commercial success represents objective evidence sufficient to rebut any *prima facie* case of obviousness.

9. Attached as Exhibit 3 is a copy of a presentation at the Biotechnology Industry Organization Meeting (BIO) held on June 26, 2001, in San Diego, California. As described therein, a "glycoprotein X" (GPX) was sent to Neose for resialylation. The purified GPX and the GPX prepared using the present process were physically characterized by eletrospray mass spectrometry. The two proteins' pharmacokinetic profiles were thereafter compared using bolus injections and continuous infusion into Cynomogus monkeys.

10. The resialyaltion was successful in restoring NeuAc on 99% of exposed Gal residues and N-linked glycans of GPX. Moreover, increasing N-linked sialylation of GPX lead to a) a 2.5 fold slower plasma clearance; and 2.2 fold increase in Css (steady-state plasma concentration) following constant infusion of 0.84 mg/Kg/d; b) 2.4 fold slower plasma clearance; and a 2.1 fold increase in elimination half life ($t_{1/2b}$) following bolus injection of 0.5 mg/kg.

11. In my opinion, this commercial success is directly related to the innovative process and thus, a nexus between the claimed invention and evidence of commercial success has been established.

12. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: October 16, 2001

David A. Zopf, M.D.

CURRICULUM VITAE

David A. Zopf, M.D.

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Social Security # 493-44-7185

Present Address Neose Technologies, Inc.
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Phone (215) 441 5890
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Education

1964 - A.B., Washington University, St. Louis, MO (Zoology Major)
1969 - M.D., Washington University School of Medicine, St. Louis, MO

Board Certification Anatomic Pathology, 1976

Brief Chronology of Employment

2000 – present	Vice President, New Product Development Neose Technologies, Inc., Horsham,
1992- 2000	Vice President, Drug Development Neose Technologies, Inc., Horsham, PA
1988-1991	Vice President and Chief Operating Officer, BioCarb Inc, Gaithersburg, MD
1982-1988	Chief, Section on Biochemical Pathology Division Cancer Biology and Diagnosis, National Cancer Institute, National Institutes of Health, Bethesda, MD
1977-1981	Expert Consultant, Laboratory of Pathology, Division Cancer Biology and Diagnosis, National Cancer Institute, National Institutes of Health, Bethesda, MD
1974-1977	Senior Staff Fellow, Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, MD
1971-1974	Clinical Associate, Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, MD

12. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: October 16, 2001

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Citizenship	United States
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Present Address	Neose Technologies, Inc. 102 Witmer Road Horsham, PA 19044 Phone (215) 441 5890 Fax (215) 441 5896

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1992- 2000	Vice President, Drug Development Neose Technologies, Inc., Horsham, PA
1988-1991	Vice President and Chief Operating Officer, BioCarb Inc, Gaithersburg, MD
1982-1988	Chief, Section on Biochemical Pathology Division Cancer Biology and Diagnosis, National Cancer Institute, National Institutes of Health, Bethesda, MD
1977-1981	Expert Consultant, Laboratory of Pathology, Division Cancer Biology and Diagnosis, National Cancer Institute, National Institutes of Health, Bethesda, MD
1974-1977	Senior Staff Fellow, Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, MD
1971-1974	Clinical Associate, Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, MD

1970-1971	Resident, Anatomic Pathology, Department of Pathology, University of Colorado School of Medicine, Denver, CO
1969-1970	Resident, Anatomic Pathology, Department of Pathology, New York University, Bellevue Medical Hospital, NY

Military Service

Active duty July 1971-1974; 1981-1988
Commissioned Corps, U.S. Public Health Service,
National Institutes of Health, Bethesda, MD

Societies

American Association for the Advancement of Science
Society for Glycobiology
Pluto Club (emeritus)
American Association of Pathologists
American Society of Biochemistry and Molecular Biology
American Chemical Society

Editorial Boards

Archives Biochemistry and Biophysics, Section on Immunochemistry and Complex Carbohydrates
Experimental Pathology
Glycoconjugate Journal (1984 - 1991)

Research Interests

Immunochemistry of complex carbohydrates
Biochemistry of human blood groups
Immune responsiveness to complex carbohydrates
Structure, function, and biosynthesis of tumor-associated antigens
Affinity methods for analysis of complex carbohydrates
Development of oligosaccharide anti-infective drugs

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Zurich, 22 February 1999

Dear Dr. Simon,

Thank you for your email message concerning antibody glycosylation. We do not anticipate any particular problems in implementing our glycosylation engineering strategy for improving antibody ADCC activity in large scale, high production cultures.

Of course, you are correct that glycosylation can be affected by process changes. In fact, we have prepared a comprehensive review of glycosylation, focusing more on genetic changes but also mentioning the process-related ones that have been reviewed recently elsewhere. I will send you a copy of this review by mail, since it is somewhat long. Unfortunately, it is available only in a book published as a conference preceding, so is not so widely accessible.

In general, I think that the process complications and costs associated with producing and utilizing a glycosyltransferase and also supplying the donor substrate make the exogenous manipulation of glycosylation generally far less attractive than engineering the cells to maximize the production of the desired glycoform. Also, you will see in the N-glycosylation pathway given in our paper that only one particular glycoform would be a suitable substrate for arriving at the desired final product, greatly reducing the potential yield of such exogenous glycosylation manipulation. Failure of much simpler cofactor-requiring enzyme catalyzed reactions to gain industrial success in competition with whole-cell biocatalysis speaks very strongly in my opinion against the competitive prospects of *in vitro* remodeling of glycosylation. I think it is much better to do this in the cells.

Best wishes,

EXHIBIT 1

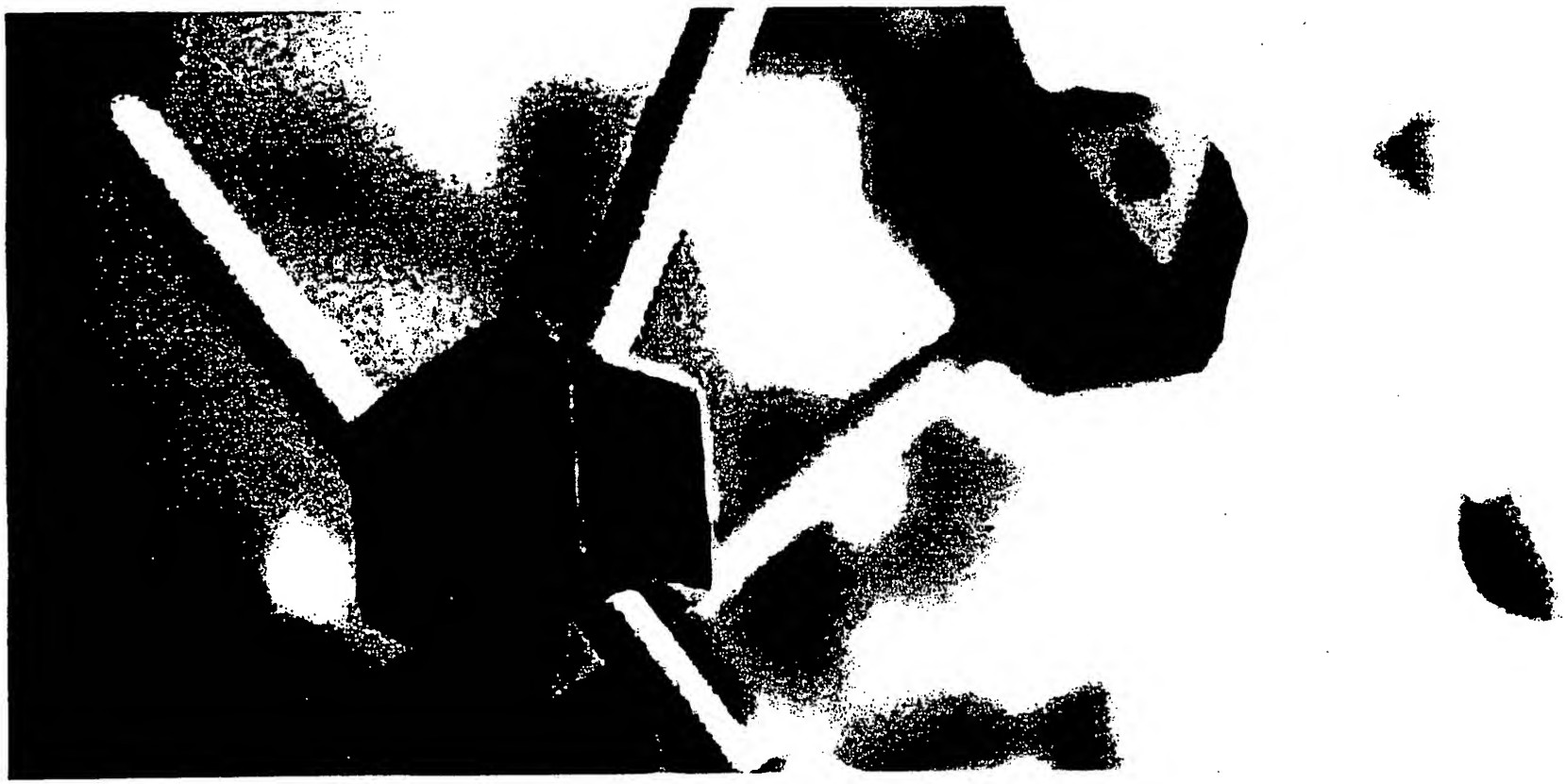
Consistent Improvement in Glycosylation

Company Product	Percent of Potential Sialylation Sites Occupied	
	Before Treatment	After Treatment
A	15%	99%
B	38%	86%
C	45%	95%
D	64%	92%
E	64%	97%
F	73%	90%
G	83%	93%
H	85%	97%

NEOSE

EXHIBIT 2

Modifying Recombinant Glycoproteins



N Jenkins, E Kattelman, D Witcher & V Wroblewski

Lilly

Answers That Matter.

EXHIBIT 3

Introduction

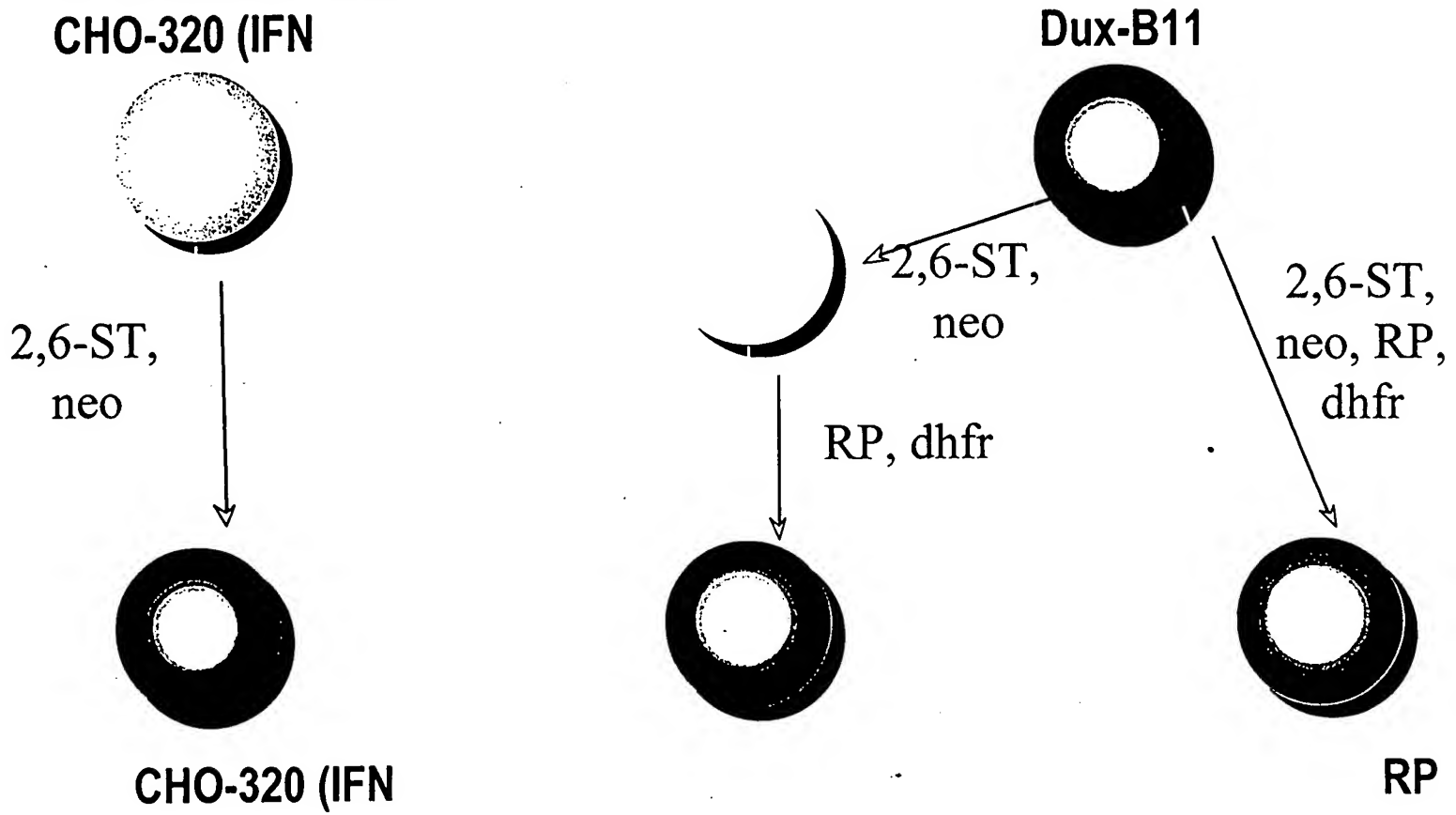
- glycoproteins are *rarely* fully glycosylated
- variants arise by incomplete biosynthesis and/or degradation by cellular glycosidases (most frequently sialidase)
- glycoform differences can lead to variable biological activity and differences in pharmacokinetics *but this is molecule-specific*
- glycoform variations also lead to lot consistency problems & are challenging analytically

Studies on Restoring Sialylation

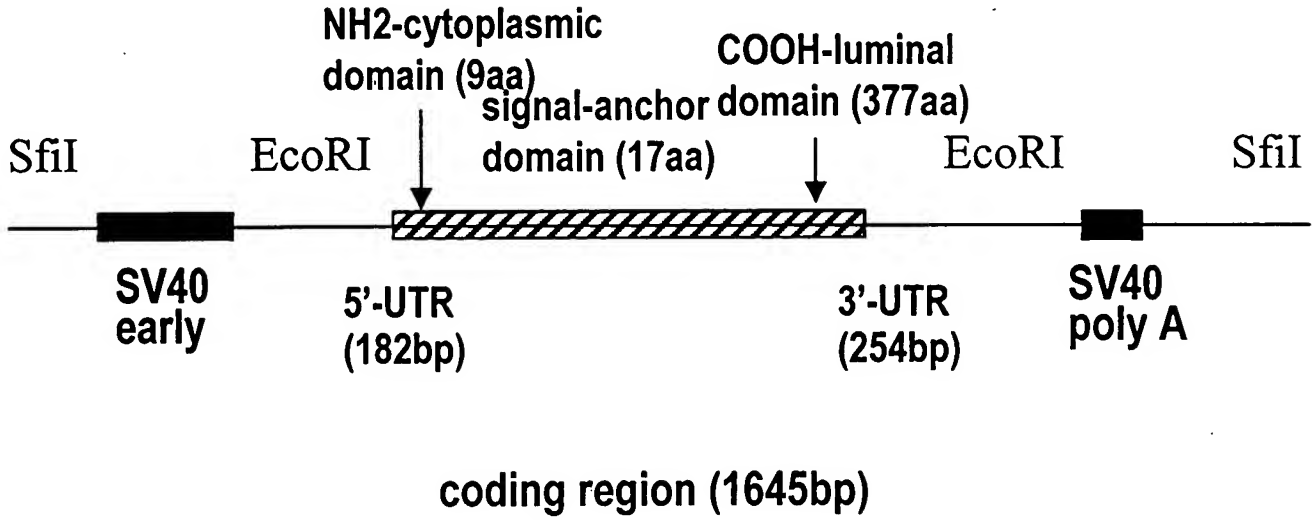
1. Transfect CHO cells with α2,6-Sialyltransferase and monitor IFN-γ glycosylation and clearance.

2. Downstream re-sialylation of GPX by α2,3-Sialyltransferase & CMP-NeuAc in vitro. Monitor GPX glycosylation and clearance.

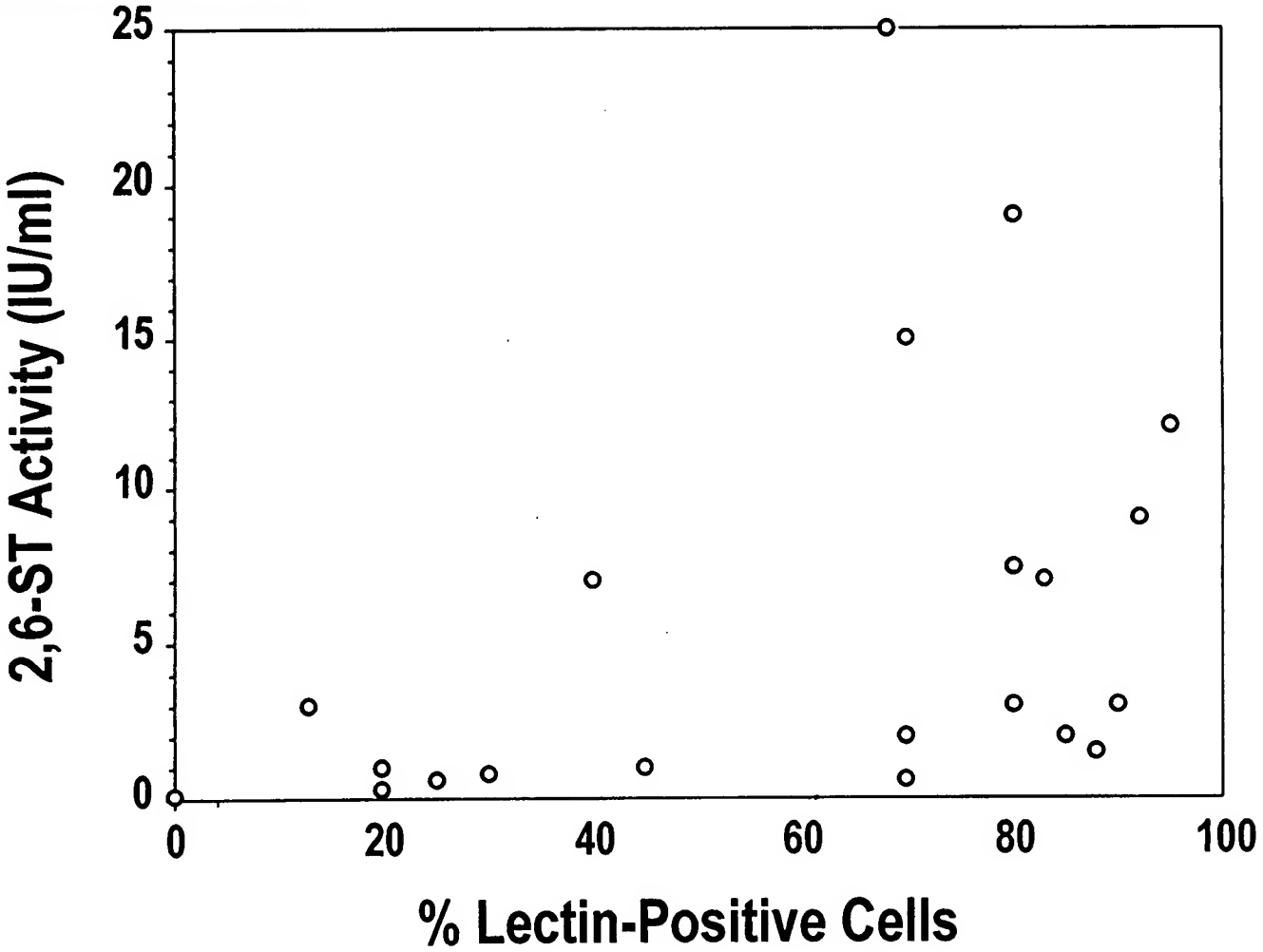
Types of Transfection



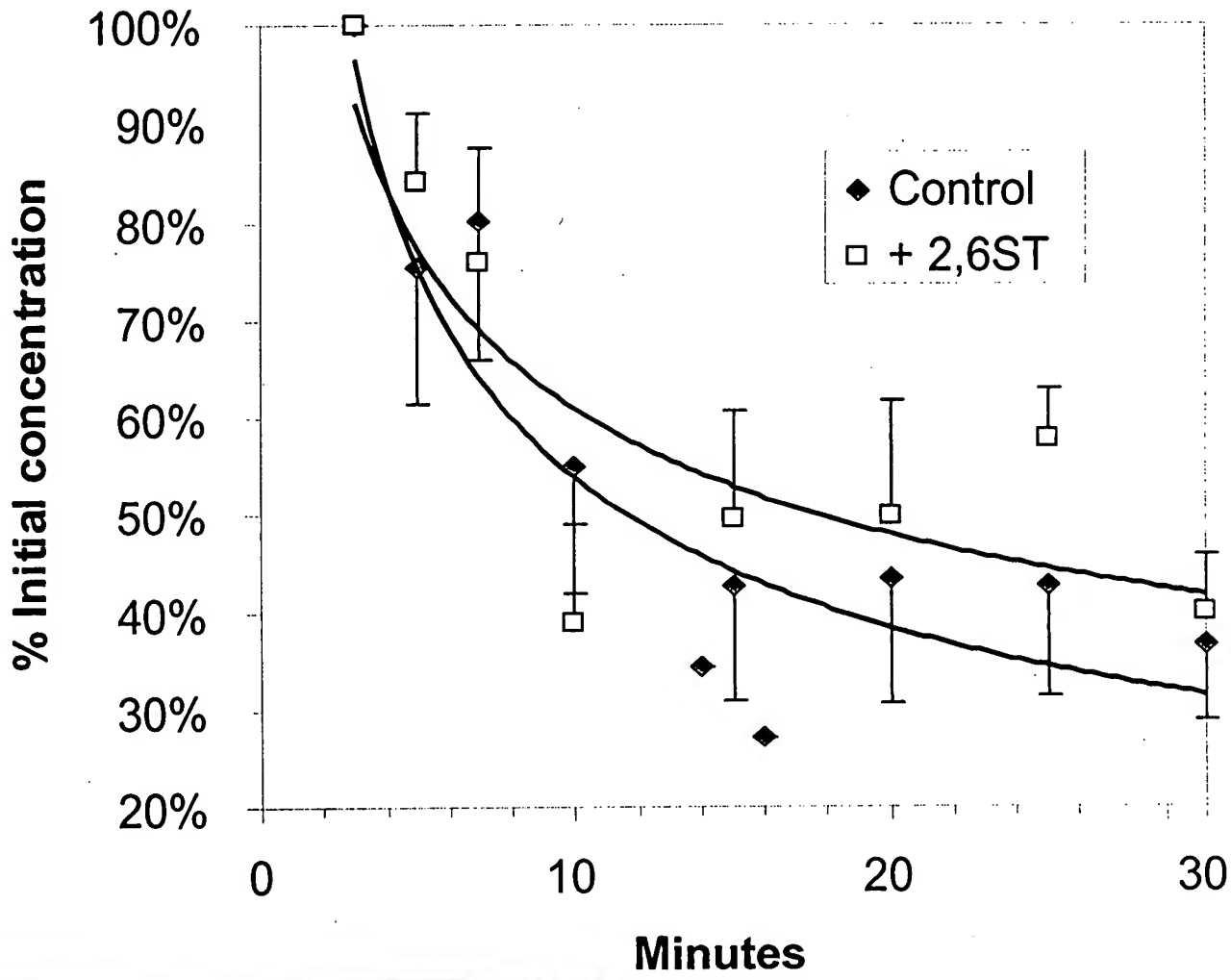
Rat α2,6-Sialyltransferase Expression Cassette (3.5 kb)



FACS & Enzyme Assay Correlation



Clearance of IFN-γ Glycoforms in Rats



Re-Sialylation Study Design

- Glycoprotein X (GPX), a protein with 1 predominantly bi-antennary N-linked glycan and 1 O-linked glycan expressed in a mammalian cell line

- One lot of purified, under-sialylated GPX was sent to Neose for re-sialylation & compared to the original purified material

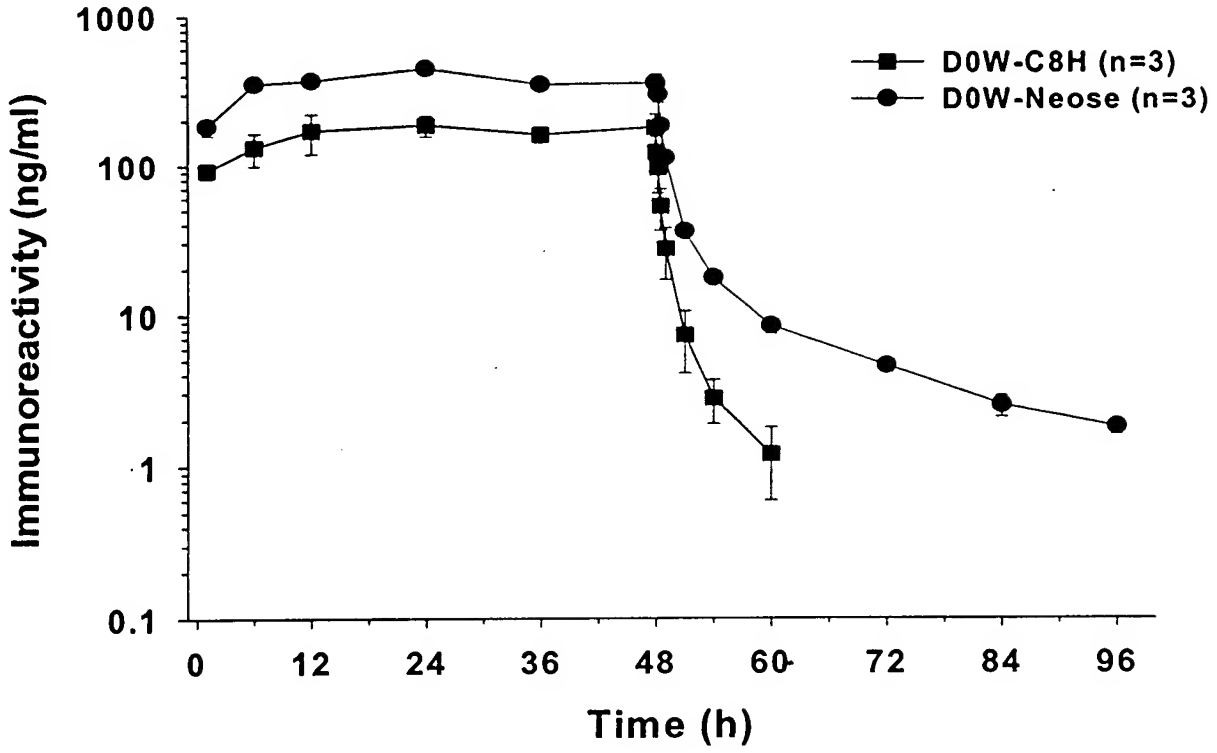
$$\text{GPX-Gal} + \text{CMP-NeuAc} \xrightarrow{\text{2,3-Sialyltransferase}} \text{GPX-Gal-NeuAc}$$

- Both types of GPX were physically characterized by ESMS & their pharmacokinetic profiles compared using bolus injections and continuous infusion into Cynomolgus monkeys

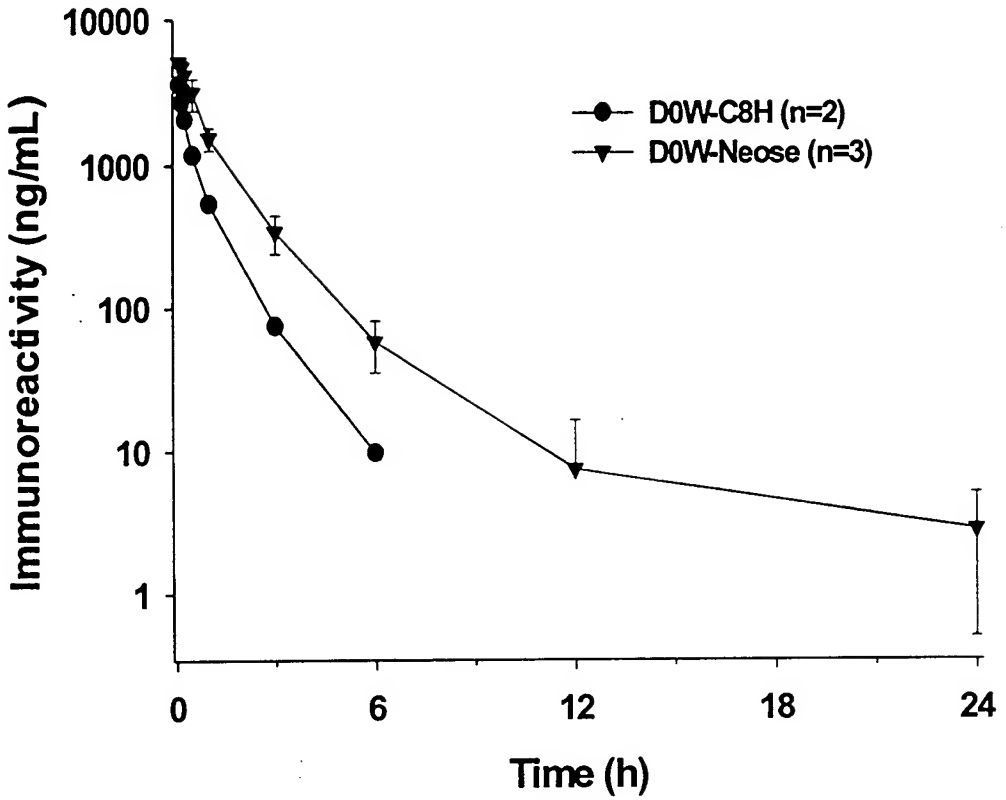
Carbohydrate Analysis by Electrospray MS

Property Sialic Acid Content (mol/mol protein)	Original material D0W-C8H	Modified material D0W-Neose	Theoretical Maximum
Total	0.84	3.12	3.79
O-Linked	0.61	0.82	1.41
N-Linked	0.23	2.30	2.38
% Sialylation			
Total	10	97	100
O-Linked	47	58	100
N-Linked	15	99	100
Under Galactosylation			
% Total	24.3	24.4	0
% O-linked	8.9	9.0	0

Continuous Infusion of 0.84 mg/Kg/d into Monkeys



Bolus Injection of 0.5 mg/Kg into Monkeys



Pharmacokinetic parameters after continuous intravenous infusion of 0.84 mg/kg/day for 48 hours

Parameter	Lot# D0W-C8H; (0.84 moles sialic acid/mole)	Lot# D0W-Neose; (3.1 moles sialic acid/mole)
Css (ng/mL)[b]	174.4 ± 32.3	380.0 ± 14.6
$AUC_{0\text{-}96\ hr}$ (ng/h/mL)	6889.5 ± 1354.7	16562.5 ± 1376.2
t1/2β (h)[c]	3.93 ± 0.38	13.5 ± 0.52
t1/2α (h)[d]	0.37 ± 0.09	0.70 ± 0.06
Clearance (L/h/kg)	0.25 ± 0.05	0.10 ± 0.01
Vss (L/kg)	0.21 ± 0.01	0.21 ± 0.03

[a] data are the mean ± standard deviation (n=3/group)
[b] Css, average of Cmax values from 12, 24, 36 and 48 hours
[c] elimination half-life
[d] distribution half-life

Conclusions

- transfection of 2,6-ST into CHO cells changes sialic acid linkages but does not result in 100% sialylation. Differences in IFN clearance were observed.

- Re-sialylation was successful in restoring NeuAc on 99% of exposed Gal residues in N-linked glycans of GPX

- this process was ineffective on non-Gal residues (incomplete substrate) and O-linked glycans (different enzyme specificity)

- increasing N-linked sialylation of GPX leads to:
  - 2.5 fold slower plasma clearance & 2.2 fold increase in Css (steady-state plasma concentration) following constant infusion of 0.84 mg/Kg/d
  - 2.4 fold slower plasma clearance & 2.1 fold increased elimination half-life (t1/2b) following bolus injection of 0.5 mg/Kg

Future Work

- evaluate purification & assay steps needed to ensure removal of Sialyltransferase & CMP-NeuAc from final GMP product
- assess scalability of enzyme steps & byproduct removal
- evaluate cost-effectiveness of Neose technology for individual proteins

PRODUCTION OF A COMPLEMENT INHIBITOR POSSESSING SIALYL LEWIS X MOIETIES BY *IN VITRO* GLYCOSYLATION TECHNOLOGY

Key Words: glycoengineering/ glycoprotein remodeling/ glycosylation/ glycosyltransferase

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Number of Article Pages: **49**

Number of Figures: **10**

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Short Title: In vitro remodeling of complement inhibitor glycans

ABSTRACT

Recombinant soluble human complement receptor type 1, sCR1, is a highly glycosylated glycoprotein intended for use as a drug to treat ischemia-reperfusion injury and other complement-mediated diseases and injuries. sCR1-sLe^x produced in the FT-VI-expressing mutant CHO cell line, LEC11, exists as a heterogeneous mixture of glycoforms, a fraction of which include structures with one or more antennae terminated by the sialyl Lewis X (sLe^x) [NeuNAcα2-3Galβ1-4(Fucα1-3)GlcNAc]) epitope. Such multivalent presentation of sLe^x was shown previously to effectively target sCR1 to activated endothelial cells expressing E-selectin. Here, we describe the use of the soluble, recombinant alpha 2-3 sialyltransferase ST3Gal-III and the alpha 1-4 fucosyltransferase FT-VI *in vitro* to introduce sLe^x moieties onto the N-glycan chains of sCR1 overexpressed in standard CHO cell lines. The product (sCR1-S/F) of these *in vitro* enzymatic glycan remodeling reactions performed at the 10 gram scale has approximately 14 N-glycan chains per sCR1 molecule, comprised of biantennary (90%), triantennary (8.5%), and tetra-antennary (1.5%) structures, nearly all of whose antennae terminate with sLe^x moieties. sCR1-S/F retained complement inhibitory activity, and in comparison with sCR1- sLe^x produced in the LEC11 cell line, contained twice the number of sLe^x moieties per mole glycoprotein, exhibited a two-fold increase in area under the intravenous clearance curve in a rat pharmacokinetic model, and exhibited a 10-fold increase in affinity for E-selectin in an *in vitro* binding assay. These results demonstrate that *in vitro* glycosylation of the sCR1 drug product reduces heterogeneity of the glycan profile, improves pharmacokinetics, and enhances carbohydrate-mediated binding to E-selectin.

INTRODUCTION

Soluble complement receptor type 1 (sCR1) is a recombinant glycoprotein which has been shown to inhibit the progression of the complement cascade in both the classical and alternative pathways by inhibiting the stable formation of C3 and C5 convertases and by serving as a cofactor in the proteolytic degradation of C3b and C4b by Factor I (Weisman *et al.,,* 1990). The administration of sCR1 has been shown to be effective in a number of animal disease models of human complement-dependent ischemia-reperfusion injury for tissues such as heart (Lazar *et al.,* 1999), liver (Lehmann *et al.*, 1998), hind limb (Kyriakides *et al.*, 2001), lung (Naka *et al.,* 1997), and intestine (Williams *et al.,* 1999). Complement inhibition by sCR1 has been shown to reduce hyperacute rejection (Pruitt *et al.,* 1997) and to enhance graft survival in many established transplant models (Kallio *et al.,* 2000, Pratt *et al.,* 1996, Stammberger *et al.,* 2000).

In some clinical situations, complement inhibition therapy could be more effective if it were targeted directly to sites of endothelial activation. At sites of inflammation, activated endothelial cells express E-selectin and P-selectin, surface adhesins with carbohydrate-binding domains that recognize the carbohydrate epitope, sLe^x (Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-) (Lasky 1995).

Previously, we have described sCR1-sLe^x (Picard *et al.*, 2000, Rittershaus *et al.,* 1999), a variant of the sCR1 glycoprotein conveniently produced in LEC11 cells transfected with the sCR1 gene. Lec11 is a mutant CHO cell line that expresses fucosyltransferase VI (FT-VI), a Golgi enzyme capable of adding fucose in α1-3 linkage to GlcNAc in oligosaccharide chains that terminate with either Galβ1-4GlcNAcβ1..., or NeuAcα2-3Galβ1-4GlcNAcβ1....(Zhang *et al.,* 1999). Of the twenty-five potential N-glycosylation sites within the sCR1 polypeptide sequence, 13-15 are occupied, the majority with biantennary chains, creating the possibility for as many as thirty sLe^x moieties per molecule of sCR1-sLe^x. However, a previously reported analysis of the N-glycans of sCR1-sLe^x showed heterologous oligosaccharides with a variety of partially sialylated and fucosylated structures yielding less than the maximal number of sLe^x moieties (12, 13). Similar heterogeneity of glycans in CHO-expressed glycoproteins has been described previously and attributed to incomplete Golgi processing, post-secretion degradation due to glycohydrolases released into cell culture media, or both (Goochee *et al.,* 1991, Jenkins *et al.,* 1996).

In this report we describe a process to introduce sLe^x moieties onto the N-glycan chains of sCR1 produced in standard CHO cell lines using *in vitro* enzymatic synthesis. This method employs serial treatment of sCR1 with soluble, recombinant rat ST3Gal-III and human FT-VI to give an sCR1-sLe^x product,

designated sCR1-S/F (for differentiation from the LEC11 product) in which the antennae of N-glycans are nearly uniformly terminated with sLe^x epitopes. The benefits of *in vitro* glycan remodeling include improved pharmacokinetics, enhanced binding to E-selectin, and a means to improve product homogeneity. Enzymatic remodeling is demonstrated at the 10 gram scale.

RESULTS

***In vitro* remodeling of sCR1 glycans.** sCR1 (250 mg) expressed in CHO cells was sialyated by treatment with ST3Gal-III plus CMP-sialic acid to give a product designated sCR1-S. After an aliquot was removed from the reaction mixture for analysis, the remaining sCR1-S was fucosylated in the same reaction vessel by the addition of FT-VI plus GDP-fucose to give a product designated sCR1-S/F. After purification by serial chromatography on ceramic hydroxyapatite and Q Sepharose, the reaction products had the same retention time and percent purity (98.5%) by RP-HPLC as the starting material, *i.e.* sCR1 (data not shown) Chemical and functional properties of these molecules were compared with those of sCR1-sLe^x, a molecule previously produced in the FT-VI-expressing Lec11 CHO cell line and shown to contain some N-linked biantennary glycans terminated with the sLe^x tetrasaccharide (Picard *et al.,* 2000).

From the mannose content of sCR1, sCR1-S, sCR1-S/F, and sCR1-sLe^x (Table I) it may be inferred that these molecules contain approximately 13-15 N-glycan chains per mol protein (assuming 3 mol mannose per N-glycan chain). The FACE oligosaccharide profile for sCR1 (Figure 1) shows 3 major bands consistent with a biantennary structure containing 0, 1 or 2 sialic acid residues, as described previously (Picard *et al.,* 2000). Monosaccharide composition of sCR1 (Table I)

suggests that approximately 57% of total galactosyl residues are substituted with sialic acid (19 mol sialic acid/ 33.2 mol galactose). By comparison, the FACE oligosaccharide profile for sCR1-S (Figure 1) shows one major band that migrates at a position consistent with a biantennary structure containing 2 sialic acid residues and monosaccharide analysis reveals the galactose/sialic acid ratio to be 1:1 (Table I).

FACE analysis of glycans from sCR1-S/F, prepared by enzymatic fucosylation of sCR-S, suggests that N-glycans are predominantly biantennary and that fucosylation at both antennae is nearly complete (Figure 1). The dominant oligosaccharide band derived from sCR1-S/F was cut out and extracted from the gel. Sequential removal of monosaccharide residues from the extracted glycoprotein using specific glycosidases gave products with mobilities consistent with α1-6 core-fucosylated, biantennary N-glycans (Figure 2). Monosaccharide analysis of sCR1-S/F shows the presence of 39.3 moles fucose per mol sCR1-S/F, a figure in agreement with the prediction from theory that 39-45 fucose residues per mol protein would be present if all N-glycans were core fucosylated and enzymatic fucosylation of antennary GlcNAc residues were complete.

The FACE oligosaccharide profile for sCR1-sLe^x, a glycoprotein produced in LEC11 CHO cells, shows at least seven bands (Figure 1) with some common to sCR1 and others shown previously (Picard *et al.,* 2000) to represent core fucosylated structures with α1-3 fucosylation at one or more antennae. Heterogeneity in the degree of fucosylation of the N-glycan chains from sCR1-sLe^x also can be appreciated from the results of monosaccharide analysis (Table I). For example, it may be calculated (assuming three mannose residues per chain) that sCR1-sLe^x contains an average of 2.5 fucosyl residues per glycan chain. By contrast, the fucose content per glycan chain increases from 0.95 for sCR1-S to 3.3 for sCR1-S/F, a result that correlates well with the single band visualized by FACE analysis of sCR1-S/F (Figure 1).

Oligosaccharide sequencing using FACE. The linkage of terminal sialic acids on sCR1-S/F was assessed by digestion with specific neuraminidases (Figure 3). Complete removal of sialic acid by treatment of band 1 from sCR1-S/F with NANase 1, indicates that sialic acid residues are α2-3 linked, as expected.

Optimization of sialylation reaction for scaleup. To establish conditions for scaleup of sialylation, sCR1 (5 mg/mL) was incubated with varying amounts of

ST3Gal-III (10, 25, 75, 100, 200, 300 and 400 U/mL) and 5 mM CMP-sialic acid plus a trace amount of radiolabeled CMP-sialic acid for 24 hours at 32°C. At an ST3Gal-III concentration of 150 mU/mL, incorporation of radiolabeled sialic acid reached 91% of maximum after 24h, and 100% at 48h. The lowest concentration of enzyme required to give near maximum incorporation (approximately 40 mol sialic acid/mol protein) under these conditions was 25 mU/mL ST3Gal-III (Figure 4). It should be noted that the contribution of triantennary and tetra antennary species may be responsible for the observation that greater than 30 moles of sialic acid was added per mole of sCR1. Increasing the CMP-sialic acid concentration from 5 mM to 10 mM did not affect the level of sialylation of sCR1 at any of the ST3Gal-III concentrations tested (data not shown). HPLC and MALDI-MS analysis of glycans released from sCR1-S revealed that at all concentrations of enzyme tested, the product contained predominantly disialylated, biantennary, core fucosylated N-glycans (data not shown). A concentration of 200 mU ST3Gal-III/mL was chosen for scaleup to ensure completeness of reaction.

Optimization of fucosylation. To establish conditions for scaleup of fucosylation, sCR1-S (5 mg/mL) was incubated with varying amounts of FT-VI (10, 20, 40, 60, 100, 220, 440 mU/mL) and 5 mM GDP-fucose plus a trace of radiolabeled GDP-fucose for 24 hours at 32°C. The lowest concentration of enzyme required to give

near maximum incorporation of fucose under these conditions was 100 mU/ml FT-VI (Figure 5). Increasing the GDP-fucose concentration from 5 mM to 10 mM did not increase fucose incorporation at several different FT-VI concentrations tested (data not shown).

For products of reactions run at all concentrations of FT-VI $\geq$ 100 mU/mL, the glycan structures identified by HPLC and MALDI-MS were almost the same and essentially indistinguishable from the structures described below for sCR1-S/F produced at the 10 gram scale.

Remodeling at 10 gram scale. Purified sCR1 (10g) was incubated first with ST3Gal-III plus CMP-sialic acid at 32°C for 36 hr, and then, following addition of FT-VI plus GDP-fucose, incubated at 32°C for another 36 hr period..

FACE analyses of glycans from sCR1, sCR1-S, and sCR1-S/F for reactions performed at the 10 g scale (data not shown) were essentially indistinguishable from FACE results obtained at the 250 mg scale (Figure 1), suggesting that occupancy of potential acceptor sites for ST3Gal-III and FT-VI on sCR1 at the 10 g scale was nearly complete.

HPLC profiles for 2AA derivatized glycans of sCR1, sCR1-S, and sCR1-S/F are shown in Figure 6 and the percentages of glycan species estimated from integrated peak areas are summarized in Table II. After *in vitro* sialylation with

ST3Gal-III, neutral glycans comprising 50% of carbohydrate chains in sCR1, are reduced to 2% of chains in sCR1-S, and monosialo glycans likewise decrease to from 35% in sCR1 to 17.5% in sCR1-S (Figure 6 and Table II). Overall, about 90% of N-glycans are biantennary and these chains contain an average of 1.8 sialic acid moieties per glycan. Among the minority of biantennary glycans on sCR1-S that are monosialylated, some lack galactose on one antenna, whereas others contain two galactosyl residues, only one of which is sialylated. The remaining 10% of glycans are fully sialylated tri-antennary (8.5%) or tetra-antennary (1.5%) structures.

After fucosylation of sCR1-S to create sCR1-S/F, HPLC and MALDI-MS analyses (Table III and Figure 7) showed that more than 95% of the glycans were fucosylated by FT-VI. About 62% of the total N-glycans gained two fucose residues, and about 30% gained a single fucose residue. Failure to accept two fucosyl residues was, in part, due to missing galactosyl residues on one or more antennae. From these results it can be estimated that the sCR1-S/F molecules created by consecutive *in vitro* sialylation and fucosylation reactions contain, on average, 28 sLe^x epitopes per protein molecule, whereas sCR1-sLe^x, glycosylated and secreted by the FT-VI-expressing Lec11 CHO cell, contains approximately 14 sLe^x epitopes per protein molecule (Table I).

To check the stability of sCR1 under conditions of incubation with glycosyltransferases, a small amount of protein was analyzed on SDS-PAGE after each remodeling reaction. There was no evidence of degradation of the polypeptide after incubation with either ST3Gal-III or FT-VI (data not shown).

Pharmacokinetics. When sCR1-S prepared at the 250 mg scale was injected intravenously into rats, the observed AUC_{last} was 2-fold greater than the AUC_{last} for sCR1 ($p<0.004$), indicating a significantly greater exposure of the more completely sialylated form of the complement inhibitor to intravascular cells following dosing (Figure 8).

***In vitro* anti-hemolytic activity.** The IH50 values for sCR1, sCR1-S, sCR1-sLe^x, and sCR1-S/F as inhibitors of human complement-mediated lysis of sheep red blood cells were found to be similar (Figure 9 and Table IV), indicating that *in vitro* glycosylation of sCR1 to yield sCR1-S or sCR1-S/F does not significantly impact the complement inhibitory properties of the molecule in the classical pathway.

***In vitro* binding to E-Selectin.** Figure 10 shows that sCR1-sLe^x and sCR1-S/F bind E-selectin in a concentration dependent manner. The IC_{50} for sCR1-sLe^x from this plot is approximately 5 nM, and for sCR1-S/F approximately 0.4 nM. The observed 10-fold increase in inhibitory potency presumably is due to enhanced avidity, attributable to the increased density of sLe^x moieties on sCR1-S/F (28/mol) as compared with sCR1-sLe^x (14 per mol) (see Table I). The specificity of this binding was demonstrated by its calcium requirement and by the observation that sCR1 (which does not contain any sLe^x structures) does not inhibit E-selectin binding at concentrations as high as 10 μM (data not shown).

DISCUSSION

sCR1, or soluble complement receptor type 1 (sCR1), made by standard CHO production methods, possesses predominantly biantennary oligosaccharides that are incompletely sialylated. We have previously described an alternately glycosylated form of sCR1 called TP20 or sCR1-sLe^x (Picard *et al.,* 2000, Rittershaus *et al.,* 1999), secreted by the FT-VI-expressing Lec11 CHO cell line and bearing sLe^x moieties on a fraction of its N-linked oligosaccharides. In this report we describe *in vitro* enzymatic remodeling of sCR1 by the stepwise application of two soluble, recombinant glycosyltransferases in "one pot": the first step adds sialic acid to make sCR1-S, and the second adds fucose to make sCR1-S/F. The product of these glycan remodeling reactions contains an average of 28 sLe^x moieties per mol, as compared with 14 per mol found in CHO cell-produced sCR1-sLe^x.

That the sCR1 protein remains intact under conditions of glycan remodeling was demonstrated by RP-HPLC and SDS-PAGE analyses showing single polypeptides with expected molecular weights for sCR1-S and sCR1-S/F. Evidence for (i) conformational stability under conditions of the *in vitro* glycosylation reactions, and (ii) preserved function despite variations in glycan structure, is provided by the observed near equivalence in bioactivity of sCR1,

sCR1-S, sCR1-S/F, and CHO-produced sCR1-sLe^x in a standard complement inhibition assay.

The oligosaccharide structures associated with sCR1-S and sCR1-S/F were assessed by a number of methods. FACE profiling demonstrated a more fully sialylated set of glycoforms for sCR1-S as compared with sCR1, and nearly homogeneous, fully sialylated and fucosylated biantennary N-glycans for sCR1-S/F. Sequencing experiments using FACE provided supporting evidence that sialic acid was linked α2-3 to galactose and that the predominant, single oligosaccharide band derived from sCR1-S/F was $BiNA_2F_2$. The analyses we performed do not establish linkages between the terminal and penultimate sugars that define sLe^x (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-) versus sLe^a (NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-). However, two factors make it likely that the glycans of sCR1-S/F do, in fact, terminate in sLe^x. First, it is known that in CHO cells, N-linked glycans are most commonly formed by β4GalT-1, and hence have the type-2 structure, Galβ1-4GlcNAcβ1- (Lee *et al.,* 2001). Second, the acceptor specificity of FT-VI is known to be restricted to type-2 chains (Costache *et al.,* 1997, Weston *et al.,* 1992).

During optimization of the sialylation reaction, we noted that incubation of sCR1 with either a low concentration of ST3Gal-III (10 mU/mL) for 24 hr or a higher concentration (75 mU/mL) for 1 hr produced a nearly maximally sialylated

product. Even after incubation at the highest concentration of sialyltransferase tested (600 mU/mL for 24H), a small fraction of monosialylated, biantennary species persisted, perhaps due to steric hindrance at particular sites. Improved pharmacokinetics observed for the fully sialylated sCR1-S molecule as compared with sCR1 is likely a consequence the added sialic acid blocking the interaction of terminal galactosyl residues with hepatic asialoglycoprotein receptors (Stockert, 1995).

We observed that FT-VI at 25 mU/mL fucosylates most sialylated biantennary glycans within 24 hr. No significant differences were observed in catalytic activitities of FT-VI expressed in the NSO versus *Aspergillus niger* expression systems. The sCR1 polypeptide was shown to be stable following prolonged incubation with enzyme from either source.

In vitro glycosylation of sCR1 at the 10 g scale was carried out at enzyme concentrations selected to ensure nearly complete reaction at each stage. Success with the single experiment reported is consistent with the ability to predict useful scaled up reaction conditions over a range of at least 40-fold based on mass of starting substrate. Both the ST3Gal-III and FT-VI enzymes used to glycosylate 10 g sCR1 were produced in *Aspergillus niger*, an expression system widely used for the manufacture of industrial enzymes in ton quantities. While further scaleup

would require refinement of incubation conditions, it can be estimated from present results that glycosylation of 1 kilogram of sCR1 might require 40,000U of ST3Gal-III, and 20,000U of FT-VI, amounts that seem plausible to produce at reasonable cost in an industrial setting.

The optimized conditions chosen for scale-up were very similar to the conditions used to generate material used for *in vivo* and *in vitro* studies. Compared with sCR1-sLe^x, sCR1-S/F was shown to have twice the number of sLe^x moieties and about a ten-fold higher apparent affinity for binding to E-selectin. This higher affinity presumably results from increased cooperativity in a multivalent binding reaction wherein sLex moieties distributed widely over sCR1-S/F engage multiple immobilized E-selectin molecules. In certain clinical situations, the anti-complement inhibitory and anti-inflammatory activity of sCR1-S/F could be effectively targeted via a similar mechanism to sites of inflammation where endothelial cells have been activated and have upregulated expression of adhesion molecules including P- and E-selectin. sCR1-sLe^x has been shown to be superior to sCR1 in a complement and selectin dependent lung injury model (Mulligan *et al.,* 1999), in a murine model of ischemic stroke (Huang *et al.,* 1999), in moderating skeletal muscle reperfusion injury (Kyriakides *et al.,* 2001), in moderation of acid aspiration injury (Kyriakides *et al.,* 2001), in reducing ischemia/reperfusion injury in rat lung grafts (Schmid *et al.,* 2001), and in a

myocardial ischemia and reperfusion model in the rat. sCR1-sLe^x significantly reduced myocardial infarct size and was significantly more effective than sCR1 in reducing neutrophil infiltration into the infarction (Zacharowski *et al.,* 1999). It will be interesting to investigate whether sCR1-S/F is even more effective than sCR1-sLe^x in similar animal models.

MATERIALS AND METHODS

Complement proteins, antibodies, enzymes and other reagents. Purified sCR1 and sCR1-sLe^x were prepared as previously described (Rittershaus *et al.,* 1999). Nucleotide sugars (CMP-sialic acid and GDP-fucose) were manufactured at Neose. CMP-sialic acid was prepared from CTP and sialic acid with recombinant CMP NeuAc synthetase (Shames *et al.,* 1991). GDP-fucose was either made from GDP-mannose using GDP-mannose 4,6-dehydratase and GDP-4-keto-6-deoxymannose 3,5-epimerase/reductase, or purchased from Yamasa Corporation (Chiba, Japan). Recombinant ST3Gal-III (rat) was expressed in *Aspergillus niger*, and purified by ion exchange chromatography on SP Sepharose (Amersham Biosciences, Piscataway, NJ). rFT-VI (human) was expressed either in NSO cells or *Aspergillus niger* as a soluble protein lacking the transmembrane domain, and purified on SP Sepharose. Soluble recombinant E-selectin was purchased from R&D Systems (Minneapolis, MN). Streptavidin-horseradish peroxidase conjugate (SA-HRP) was from Pierce (Rockford, IL), and biotinylated-polyacrylamide polymer (PAA-sLe^x) was from GlycoTech Corporation (Rockville, MD). Anti-sCR1 monoclonal antibodies 6B1.H12 and 4D6.1 were prepared as previous described (Nickells *et al.,* 1998). Standards and glycosidases used in FACE™ analyses were from GLYKO (Novato, California).

Preparation of sialylated sCR1 (sCR1-S). 250 mg lyophilized sCR1 was reconstituted and buffer exchanged into 50 mM Tris, 0.15M NaCl, 0.05% NaN_3, pH 7.2 using gel filtration columns (PD-10, Amersham Biosciences) and the concentration of sCR1 was adjusted to 5 mg/mL with the same buffer. Following addition of ST3Gal-III (150 mU/mL) and CMP-sialic acid (7 mM) the mixture was incubated at 32°C. A separate aliquot of the reaction mixture to which a trace amount of CMP-[^{14}C]sialic acid was added was incubated in parallel. From this aliquot samples were withdrawn at various times and fractionated by isocratic HPLC/size exclusion chromatography at 0.5mL/min in 45% MeOH, 0.1%TFA (7.8mm x 30 cm $TSKG2000_{SWXL}$ column, particle size 5 μm, TosoHaas). Incorporation of sialic acid into glycoprotein was calculated from the fraction of counts in the first eluted peak and the known concentration of sugar nucleotide.

Preparation of sialylated, fucosylated sCR1 (sCR1-S/F). After the above sialylation reaction had proceeded for 48 hr, GDP-fucose was added to a final concentration of 7 mM, $MnCl_2$ to 5 mM, and rFT-VI to 0.1 U/mL. A trace amount of GDP-[^{14}C]fucose was added to a separate aliquot and both reaction mixtures were incubated at 32°C. Chromatography of the radiolabeled mixture as described

above showed the transfer of approximately 44 moles/mole sCR1-S after 48H, and 47 moles after 48H. The product was provisionally designated sCR1-S/F.

Removal of nucleotide sugars and residual glycosyltransferases using ceramic hydroxyapatite and Q Sepharose chromatography. Glycosyltransferases and nucleotide sugars were removed from remodeled sCR1-S and sCR1-S/F by chromatography on ceramic hydroxyapatite (CHT, Type I; BioRad, Hercules, CA) followed by Q Sepharose (Amersham Biosciences). Purity was assessed by RP-HPLC on a Poros R1/10 column (4.6 mmD/100 mmL, Applied Biosystems, Framingham, MA).

Optimization of sialylation and fucosylation reactions prior to scaleup. sCR1 was thawed slowly at 4°C and buffer exchanged into 50mM Tris-HCl pH 7.5, 150mM NaCl, using a PD10 column. *In vitro* sialylation of sCR1 (5mg/mL) was evaluated using varying amounts of ST3Gal-III, 5mM CMP-sialic acid, in the presence of 0.02% sodium azide at 32°C for 24h. A trace amount of CMP-[^{14}C]sialic acid was added to an aliquot to monitor incorporation of radioactive sialic acid as described above.

To the product (sCR1-S) of the above reaction performed at a sialyltransferase concentration of 100 mU/mL, (still containing the sialylation

reagents), was added $MnCl_2$ and GDP-fucose, each to a final concentration of 5 mM, varing amounts of FT-VI, and a trace amount of GDP-[^{3}H]fucose. The resulting reaction mixture was incubated at 32°C for 24 hr. Incorporation of radioactive fucose into the product (sCR1-S/F) was monitored as described as above for sialic acid.

sCR1 Remodeling at 10 g scale. Purified sCR1 (10 g) was dialyzed exhaustively at 4°C against 50 mM Tris HCl, 0.15M NaCl, pH 7.5, adjusted to a concentration of 5 mg/ml with the same buffer, and incubated with ST3Gal-III (200 mU/ml) and CMP-sialic acid (5 mM) for 36 hr at 32°C. After 36 h, an aliquot containing the sialylated product (sCR1-S) was withdrawn for analysis and the following reagents (final concentrations) were added: rFT-VI (100 mU/mL), GDP-Fucose (5 mM), $MnCl_2$, (5 mM). After further incubation at 32°C for 36 h, a precipitate (manganese phosphate) was removed by centrifugation at 3000g for 5 min, and the sialylated and fucosylated product (sCR1-S/F) was stored at -70°C.

Monosaccharide analysis by HPLC. The neutral and amino sugar composition of glycoproteins was determined after trifluoroacetic acid hydrolysis and reductive amination with anthranilic acid by C18 reverse phase HPLC with fluorescence detection (Anumula 1994). Sialic acid content was determined after sodium

bisulfate hydrolysis and reaction with o-phenylenediamine by C18 reverse phase HPLC with fluorescence detection (Anumula 1995).

Carbohydrate analysis by fluorophore assisted carbohydrate electrophoresis (FACE). Carbohydrate sequencing and electrophoresis by FACE (Glyko Inc. Novato, CA and ProZyme, San Leandro, CA) was performed as previously described (Picard *et al.,* 2000).

Carbohydrate analysis by 2-AA HPLC and MALDI-TOF. Glycans were released by PNGaseF and labeled with 2-AA (2-anthranilic acid) according to the method described by Anumula and Dhume (1998) except that the labeled glycans were purified on cellulose cartridges (Glyko) according to the manufacturer's instructions. 2-AA labeled N-glycans were analyzed using a Shodex Asahipak NH_2P-50 4D amino column (4.6 mm x 150 mm). The two solvents used for the separation were A) 2% acetic acid and 1% tetrahydrofuran in acetonitrile and B) 5% acetic acid, 3% triethylamine and 1% tetrahydrofuran in water. The column was eluted isocratically with 70% A for 2.5 min, followed by a linear gradient from 70% to 5% A over a period of 97.5 min, and a final isocratic elution with 5% A for 15 min. Eluted peaks were detected using fluorescence detection with an excitation wavelength of 230 nm and an emission wavelength of 420 nm.

For MALDI analysis, a small aliquot of the AA-labeled N-glycans was dialyzed for 45 min on an MF-Millipore membrane filter (0.025 μm pore, 47 mm diameter) floating on water. The dialyzed aliquot was dried in a vacuum centrifuge, redissolved in a small amount of water, and mixed with a solution of 2, 5-dihydroxybenzoic acid (10 g/L) dissolved in water/acetonitrile (50:50). The mixture was dried onto the target and analyzed using an Applied Biosystems DE-Pro MALDI-TOF mass spectrometer operated in the linear/negative-ion mode. Glycan structures were assigned based on the observed mass-to-charge ratio and literature precedence. No attempt was made to fully characterize isobaric structures.

SDS-PAGE. sCR1 samples before and after in vitro enzymatic remodeling were separated on 8-16% gradient Tris-glycine polyacrylamide gels and stained with colloidal blue Coomassie stain. Gels, staining solutions and molecular weight standards were obtained from Invitrogen.

Assays of complement regulatory activity. The inhibition of complement-mediated lysis of antibody-sensitized sheep erythrocytes (classical pathway) was assessed as previously described (Scesney *et al.,* 1996).

E-Selectin binding assay. E-selectin binding assays were performed according to previously reported methods (Weitz-Schmidt *et al.*, 1996). Flat bottom 96 well microtiter plates were coated with 5 μg/mL recombinant human E-selectin (R&D Systems) in 150 mM NaCl, 1 mM $CaCl_2$, 20 mM Hepes, pH 7.4 (HBS). Coated wells were blocked with 2% BSA/HBS. Varying concentrations of sCR1 or sCR1-sLe^x were added to the plate. A complex of a biotinylated polyacrylamide polymer containing sLex (PAA-sLex, GlycoTech Corp.) and streptavidin conjugated horseradish peroxidase (SA-HRP) was prepared. A dilution of this conjugate complex was added to the wells containing sCR1 or sCR1-sLe^x or buffer and incubated for 90 minutes at room temperature. The wells were washed with HBS/$CaCl_2$ and TMB substrate (KPL) was added to each well. Color was allowed to develop for 15 minutes and the reaction was stopped with 2.0N H_2SO_4. Bound PAA-sLe^x complex was measured by determining the absorbance at 450 nm with a microplate reader (Molecular Devices).

Pharmacokinetic analysis in rats. Male Sprague-Dawley rats (approx. 250 gm), with indwelling jugular vein cannulas were purchased from Taconic (Germantown, NY) or Harlan Sprague Dawley (Indianapolis, IN). The catheters were periodically

flushed with 0.9% saline followed by either heparinized glycerol (1:4 glycerol / 333IU heparin/ml) or heparinized saline (333 IU/ml) to ensure patency.

Animals were injected with sCR1 or sCR1-S (10 mg/kg) via the lateral tail vein, as a bolus at time 0. Blood samples were obtained at timed intervals from the jugular vein cannula. The levels of sCR1 and sCR1-S present in the plasma samples were measured by a previously described ELISA (Rittershaus *et al.*, 1999). Briefly, microtiter plates were coated with anti-sCR1 monoclonal antibody 6B1.H12 and captured sCR1 from a sample was detected with a horseradish peroxidase conjugated anti-sCR1 monoclonal antibody 4D6.1. Pharmacokinetic data was analyzed using WinNonlin (Pharsight, Mountain View, CA).

ACKNOWLEDGMENTS

The authors would like to thank Michelle Richardson and Gang Yan for expert technical assistance.

ABBREVIATIONS

2-AA, 2-anthranilic acid; AUC, Area Under the Curve; β4GalT-1, β4-galactosyltransferase-I; CHO, Chinese hamster ovary; CMP, cytidine 5'-monophosphate; ELISA, enzyme-linked immunosorbent assay; FACE, fluorophore-assisted carbohydrate electrophoresis; FT-VI, fucosyltransferase VI; GDP, FucaseIII, fucosidase III; GalaseIII, balactosidase III; guanosine 5'-diphosphate; HBS, Hepes-buffered saline; PAA- sLe^x, MALDI-TOF MS, Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectrometry; NANase III, neuraminidase III; NSO, murine cell line (Lonza Biologics); RP-HPLC, reversed phase high pressure liquid chromatography; sialyl Lewis X biotinylated-polyacrylamide polymer; SA-HRP, streptavidin-horseradish peroxidase conjugate; sCR1, recombinant soluble human complement receptor type 1; sCR1-S, sialylated sCR1; sCR1-S/F, sialylated and fucosylated sCR1; sCR1- sLe^x , sCR1 expressed in LEC11 cells and bearing sialyl Lewis X; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; sLe^a, sialyl Lewis A; sLe^x, sialyl Lewis X; ST3Gal-III, Galβ1-3(4)GlcNAc α2-3-sialyltransferase; TMB, 3, 3', 5, 5' – Tetramethylbenzidine;

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FIGURE LEGENDS

Figure 1. Face profiling of oligosaccharides from sCR1-Lex and sCR1 before and after enzymatic remodeling: (1) Glyko oligosaccharide standard ladder, (2) sCR1, (3) sCR1-S, (4) sCR1-S/F, (5) sCR1-sLe^x. The oligosaccharide profile of sCR1 (lane 2) contains predominantly bands representing biantennary structures with two sialic acids (bottom band), one sialic acid (middle band) and no sialic acids (top band).

Figure 2. FACE analysis of oligosaccharides from sCR1-S/F after serial treatment with glycosidases . The dominant oligosaccharide band derived from sCR1-S/F (lane 2) was cut out and extracted from the gel. The resulting oligosaccharide preparation was digested sequentially remove each monosaccharide residue starting at the terminal sialic acid residue and ending at the trimannosyl core: (1) Glyko oligosaccharide standard ladder; (2) total N-linked oligosaccharides of sCR1-S/F; (3) purified dominant band (Band 1) from lane 2; (4) Band 1 treated with NANaseIII (cleaves α 2-3,4,6,8 &9 linked sialic acid); (5) Band 1 treated with NANaseIII and FucaseIII (cleaves α1-3 & 4 fucose); (6) Band 1 treated with NANaseIII, FucaseIII and GalaseIII (cleaves terminal galactose); (7) Band 1

treated with NANaseIII and FucaseIII, GalaseIII and hexosaminidase; 8. Standard trimannosyl core N-glycans with (upper band) and without α 1-6 fucose.

Figure 3. FACE analysis of oligosaccharides from sCR1-S/F treated with sialidases. The dominant oligosaccharide band (Band 1) derived from sCR1-S/F (see Figure 2, lane 2) was cut out and extracted from the gel. The resulting oligosaccharide preparation was subjected to enzymatic digestion to remove terminal sialic acid: (1) Glyko oligosaccharide standard ladder; (2) Band 1 from sCR1-S/F ; (3) Band 1 treated with NANaseI (cleaves α 2-3 linked sialic acid); (4) Band 1 treated with NANaseIII (cleaves α 2-3,4,6,8 & 9 linked sialic acid).

Figure 4. Incorporation of sialic acid into sCR1 at increasing concentrations of ST3Gal-III in a 24 hr reaction. The moles of sialic acid added are estimated from incorporation of radiolabelled CMP-sialic acid. Incorporated radiolabel is separated from free by gel filtration on a TosoHaas $TSKG2000_{SWXL}$ column.

Figure 5. Incorporation of fucose into sCR1-S at increasing concentrations of FT-VI in a 24 hr reaction. The moles of fucose added are estimated from

incorporation of radiolabelled GDP-fucose. Incorporated radiolabel is separated free by gel filtration on a TosoHaas $TSKG2000_{SWXL}$ column.

Figure 6. RP-HPLC analysis of 2AA-oligosaccharides before and after enzymatic remodeling at the 10 g scale: (A) sCR-1; (B) sCR1-S (C) sCR1-S/F. MALDI-TOF/MS analysis (data not shown) of 2AA-oligosaccharides from sCR1-S (B) indicated that: peak (a) contains monosialylated biantennary glycans that lack terminal galactose on one antenna; peak (b), constituting 12% of biantennary glycans, contains biantennary glycans with two galactose residues, but only one sialic acid; peaks (c) and (d) contain disialylated, biantennary glycans, with and without core fucose, respectively.

Figure 7. MALDI analysis of total glycans from (A) sCR1, (B) sCR1-S, and (C) sCR1-S/F remodeled at the 10g scale. The blue square is GlcNAc, the yellow filled circle is mannose, the green filled triangle is fucose, the red filled diamond is galactose, and the asterisk is sialic acid.

Figure 8. The concentration of sCR1 and sCR1-S in plasma at various time-points following bolus i.v. injection in rats.

Figure 9. Inhibition of red cell lysis via the classical pathway as a function of the concentration of sCR1 , sCR1-S, sCR1-sLe^x, and sCR1-S/F.

Figure 10. Inhibition of PAA-sLe^x binding to E-selectin coated microtiter plates in the presence of varying concentrations of sCR1-sLe^x or sCR1-S/F.

Table I

Monosaccharide Content (mol/mol glycoprotein) by HPLC Analysis

	sCR1	sCR1-S	sCR1-S/F	sCR1- sLe^x
Glucosamine	62.2	48.2	47.6	62.3
Galactose	33.2	27.5	26.7	38.3
Mannose	44.4	39.1	35.4	39.7
Fucose	15.5	12.4	39.3	33.2
Sialic acid	19	30	28	27
sialic acid/galactose	0.57	1.09	1.06	0.70
glycosylation sites/sCR1	14.8	13.0	11.8	13.2
Estimated sLe^x/sCR1-sLe^x *	n.a.	n.a.	28	14

*estimated sLe^x / sCR1-sLe^x = (Fuc/ sCR1-sLe^x - Sites/ sCR1-sLe^x) x Sial/Gal ratio

Table II. HPLC data summary of large scale remodeling

Glycan Species	Native protein (sCR1)	Sialylated protein (sCR1-S)	Sialylated and fucosylated protein (sCR1-S/F)
Neutral	50.5%	2.0%	4.0%
1 charge	35.0%	17.5%	25.5%
2 charges	13.0%	70.5%	68.5%
3 charges	1.5%	8.5%	1.5%
4 charges	ND*	1.5%	0.5%

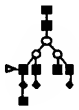
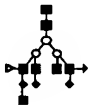
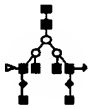
* Not detected

Table III; sCR1-S/F Glycans

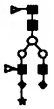
Neutral glycans

structure	%
	0.64
	0.16
	0.44
	0.43
	0.18
	0.95
	0.28
	0.35
	0.58

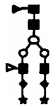
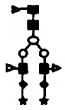
Neutral glycans

structure	%
	0.16
	0.13
	0.38
	1.51
	0.82

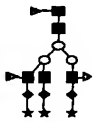
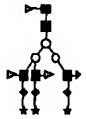
Monosialo glycans

structure	%
	0.70
	3.47
	1.06
	1.15
	7.07
	2.93
	6.12

Disialo glycans

structure	%
	17.26
	51.24

Trisialo glycans

structure	%
	0.75
	0.75

Blue squares represent N-acetylglucosamine, yellow circles represent mannose, green triangles represent fucose, red diamonds represent galactose, and black asterisks represent sialic acid.

Table IV. Anti-Hemolytic activity of Modified sCR1 and sCR1-sLe^x.

	IH50
sCR1	0.41 nM
sCR1-S	0.48 nM
sCR1-sLe^x	0.59 nM
sCR1-S/F	0.59 nM

Figure 1

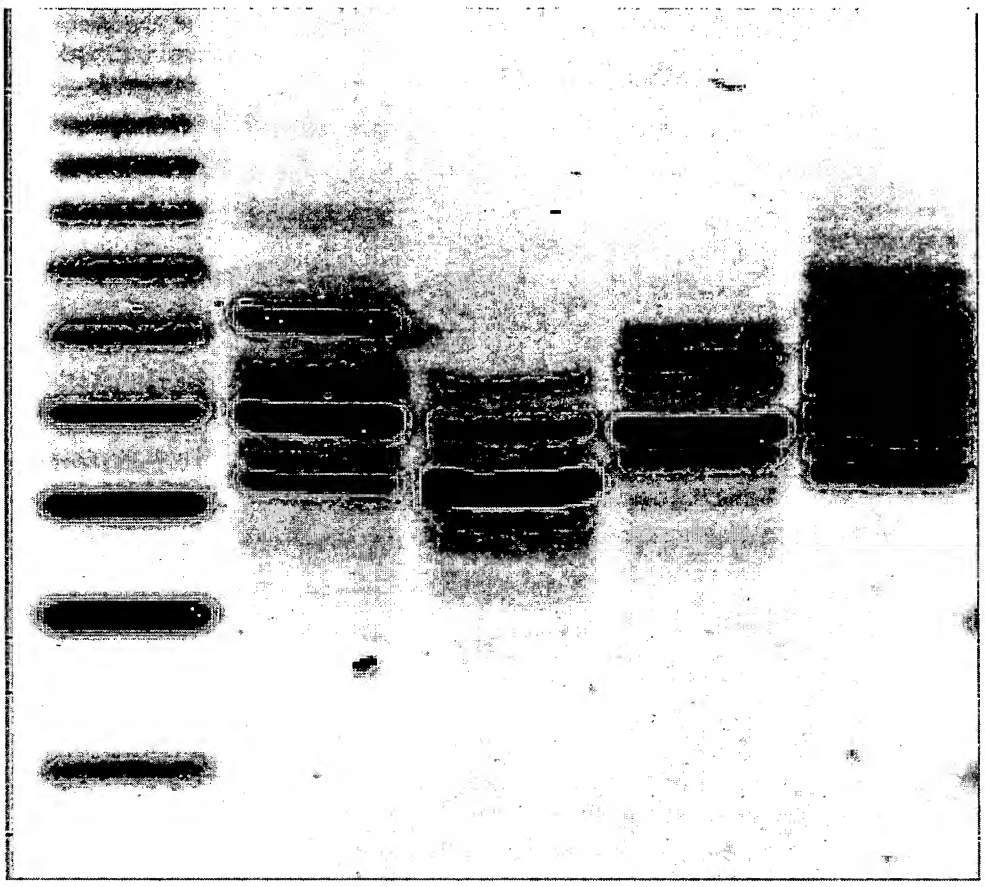
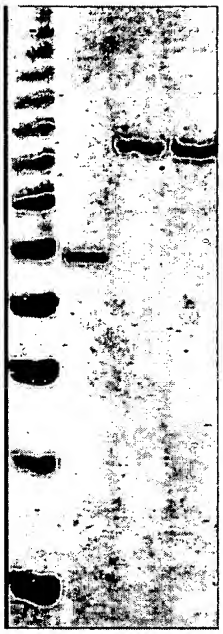


Figure 2

Figure 3



1 2 3 4

Figure 4

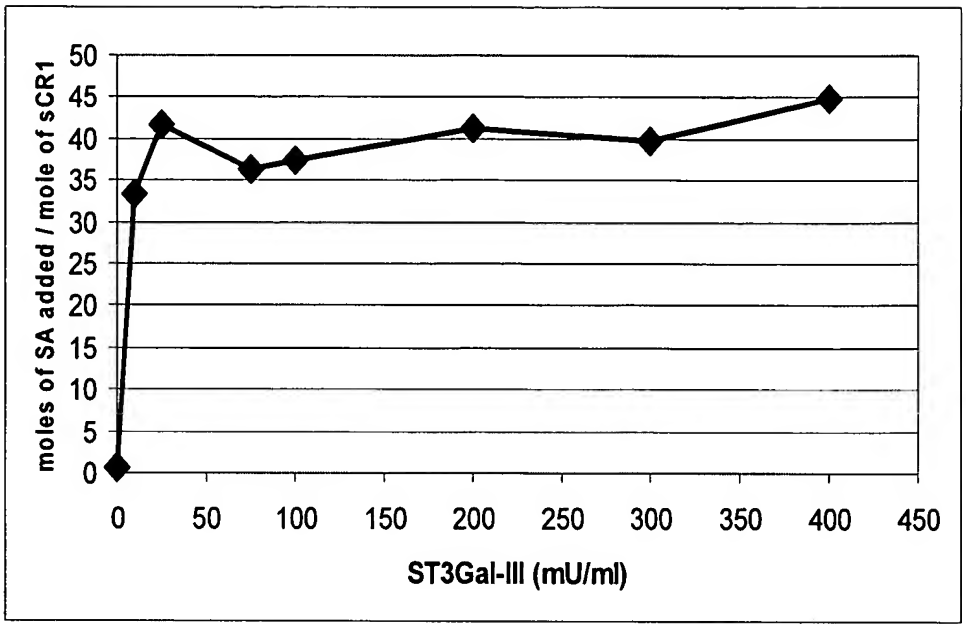

moles of SA added / mole of sCR1
50
45
40
35
30
25
20
15
10
5
0
0
50
100
150
200
250
300
350
400
450
ST3Gal-III (mU/ml)

Figure 5

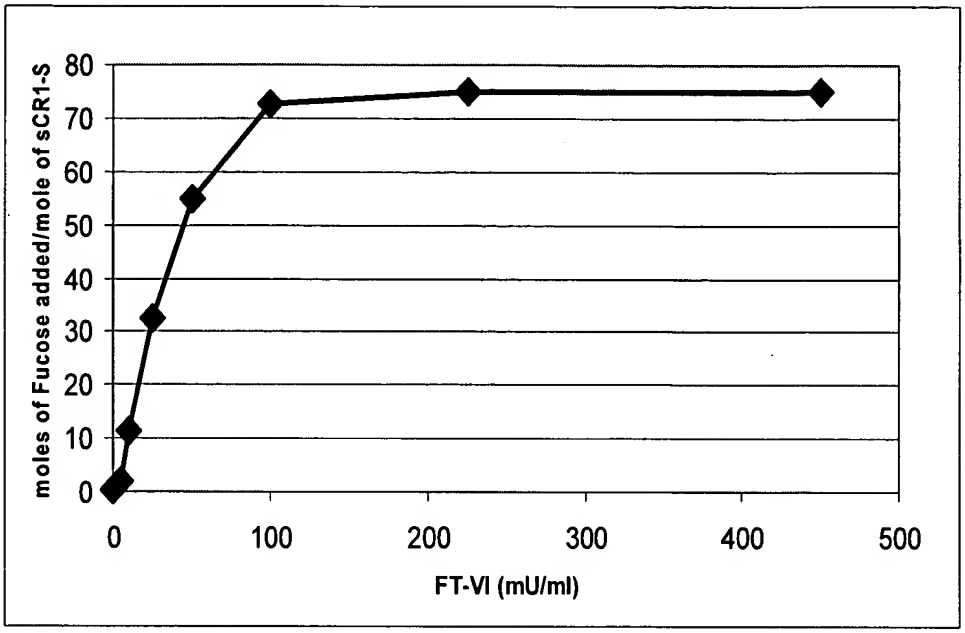

moles of Fucose added/mole of sCR1-S
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
FT-VI (mU/ml)

Figure 6

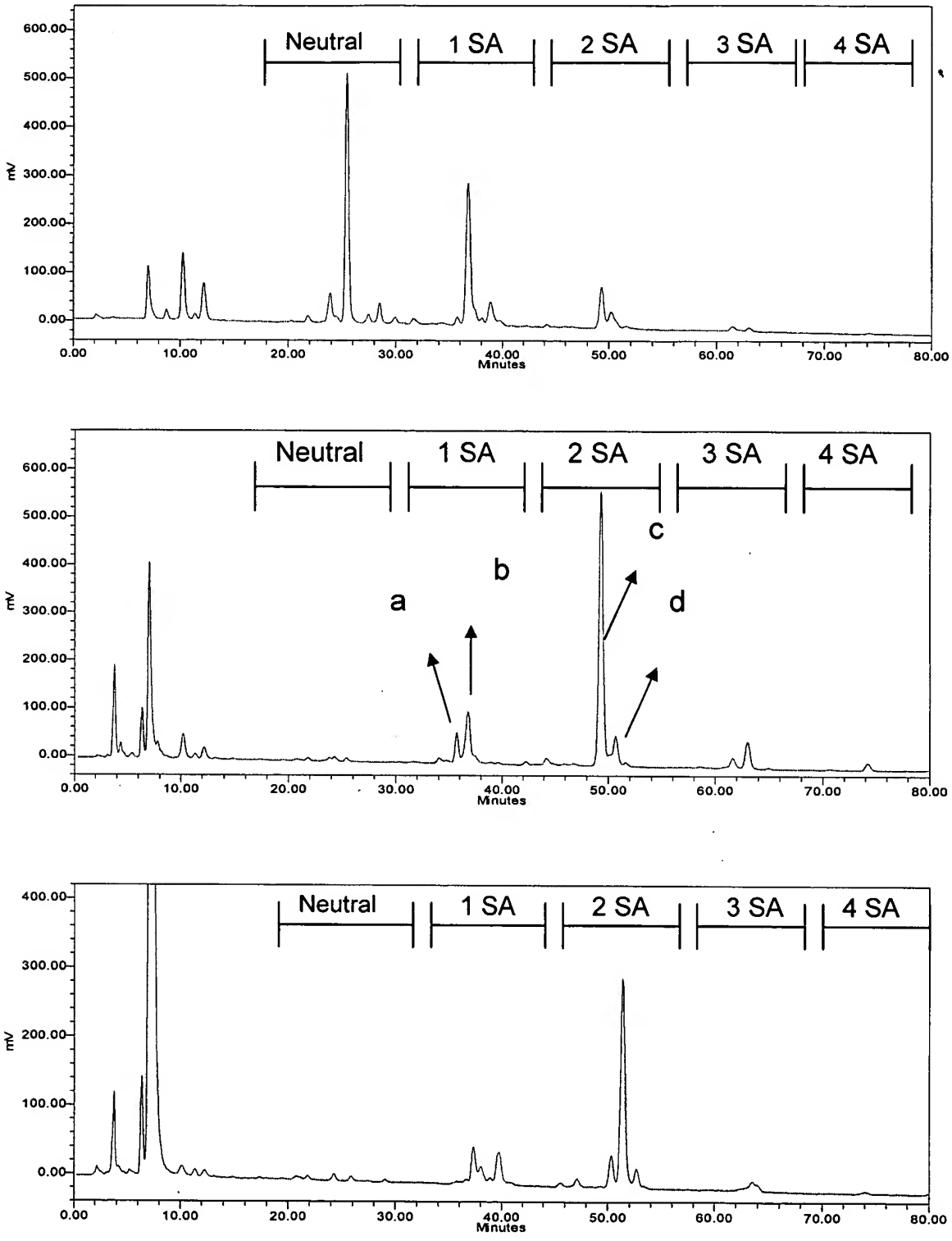


Figure 7

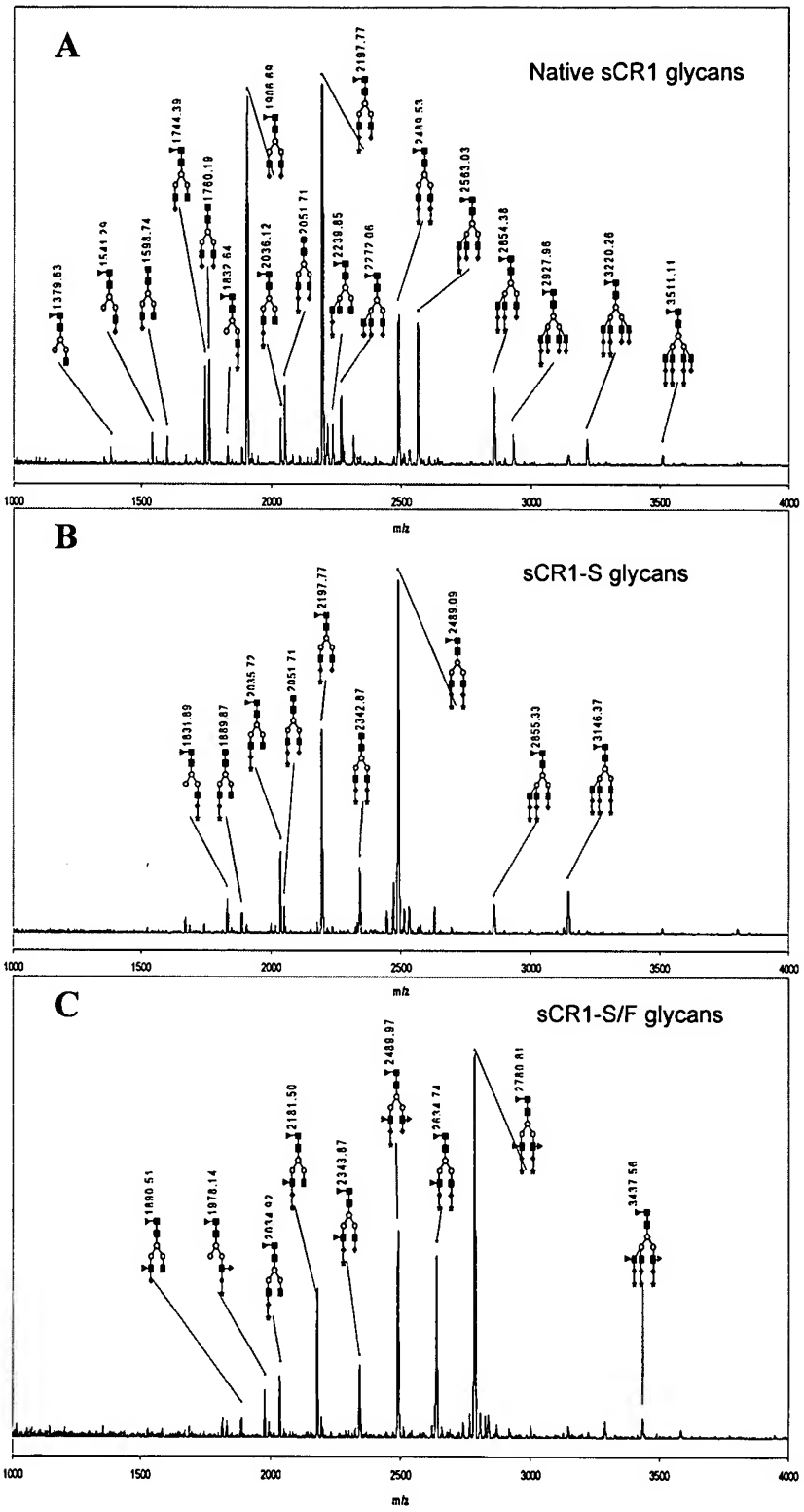

A
Native sCR1 glycans
1379.63
1744.39
1760.19
1839.64
2036.12
2051.71
2197.77
2239.85
2777.06
2563.03
2654.38
2827.98
3220.28
3511.11
1000
1500
2000
2500
3000
3500
4000
m/z
B
sCR1-S glycans
1831.89
1889.87
2035.72
2051.71
2197.77
2342.87
2489.09
2655.33
3146.37
1000
1500
2000
2500
3000
3500
4000
m/z
C
sCR1-S/F glycans
1880.51
1978.14
2014.02
2181.50
2343.87
2489.97
2654.74
2780.81
3437.56
1000
1500
2000
2500
3000
3500
4000
m/z

Figure 8; Pharmacokinetics in Rats

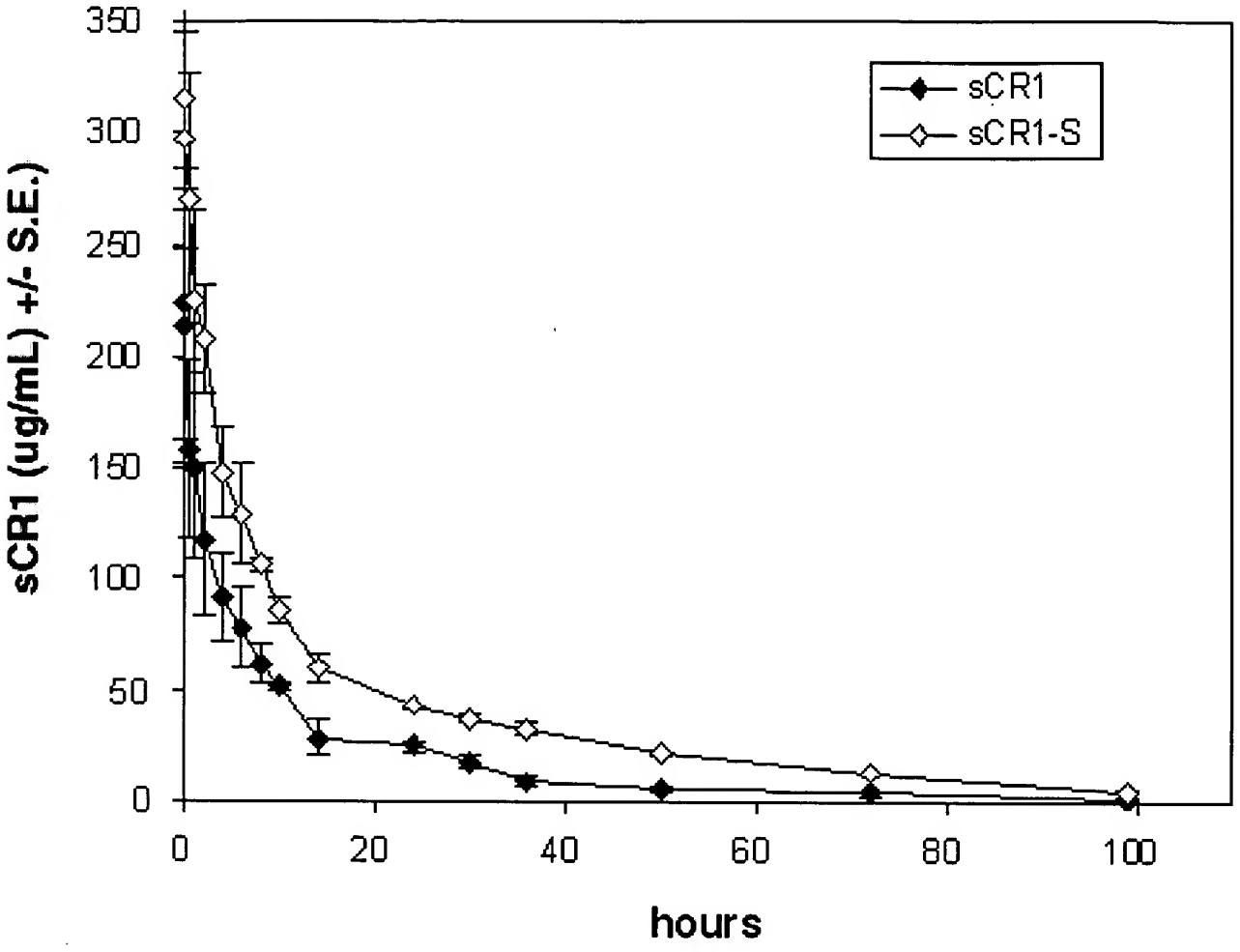


Figure 9

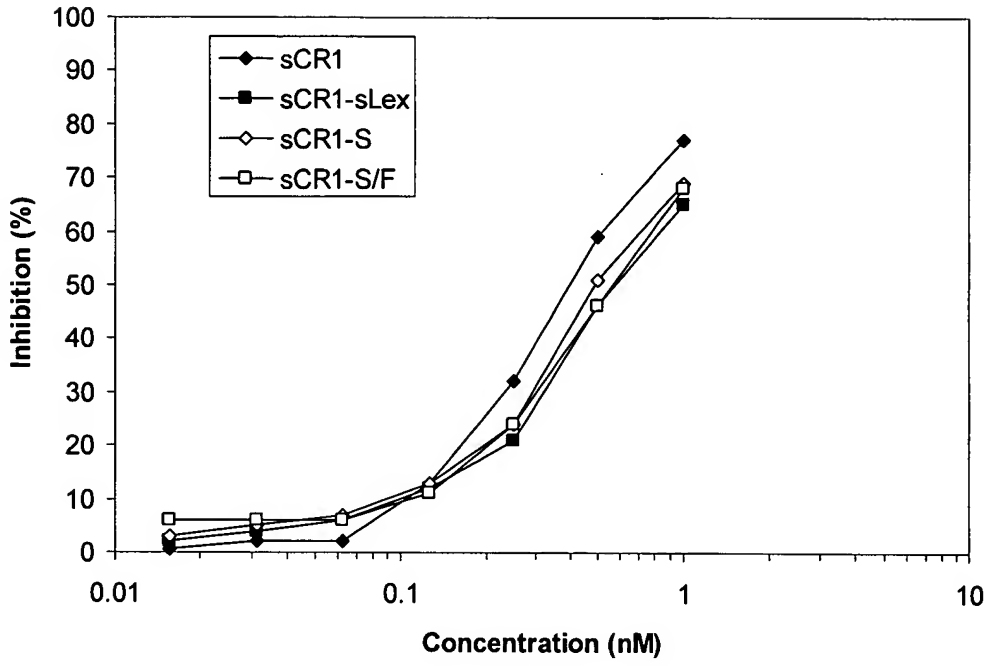
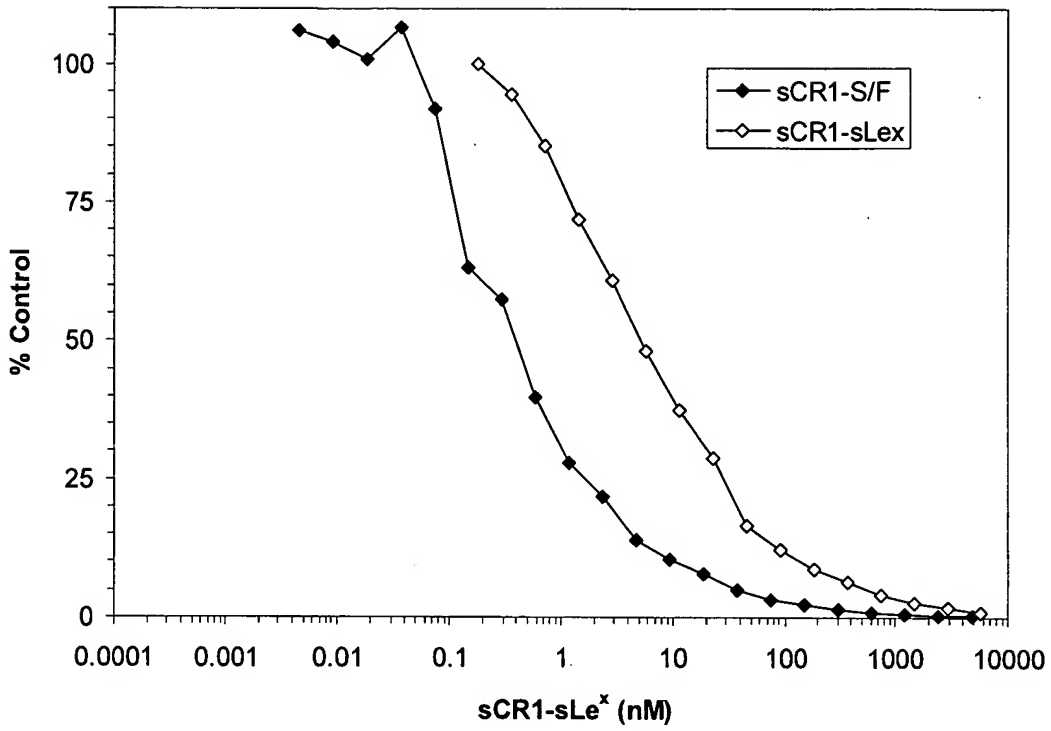

100
90
80
70
60
50
40
30
20
10
0
Inhibition (%)
sCR1
sCR1-sLex
sCR1-S
sCR1-S/F
0.01
0.1
1
10
Concentration (nM)

Figure 10


100
75
50
25
0
% Control
0.0001
0.001
0.01
0.1
1
10
100
1000
10000
sCR1-sLe^x (nM)
sCR1-S/F
sCR1-sLex

Neose Technologies and MacroGenics Sign Research Collaboration and License Agreement

HORSHAM, Pa., and ROCKVILLE, Md., April 26 /PRNewswire-FirstCall/ -- Neose Technologies, Inc. (Nasdaq: NTEC) and MacroGenics Inc. announced today that the companies have entered into a research collaboration and license agreement on multiple monoclonal antibodies. Neose will apply its GlycoAdvance(TM) and GlycoPEGylation(TM) technologies to MacroGenics compounds with the goal of improving the therapeutic properties of these proteins.

MacroGenics has the right to take a limited number of remodeled compounds into development. Following the initial research phase, MacroGenics will be responsible for funding the further development of these licensed compounds under an exclusive license from Neose. In exchange, Neose will be entitled to receive various option fee, milestone, and royalty payments as products are developed and commercialized under the agreement.

"We are impressed with MacroGenics' expertise in the antibody field and look forward to working with them. They have important new technology for the development and modification of monoclonal antibodies, particularly in the Fc region, and we believe that combining our technologies may yield more effective new treatments for chronic diseases," said C. Boyd Clarke, Neose president, chief executive officer and chairman.

"We are excited by the potential therapeutic improvements that can be made to our monoclonal antibodies utilizing Neose's GlycoAdvance and GlycoPEGylation technologies," said Scott Koenig, M.D., Ph.D., president and chief executive officer of MacroGenics.

Neose is a biopharmaceutical company focused on the improvement of protein therapeutics through the application of its proprietary technologies. By applying its GlycoAdvance and GlycoPEGylation technologies, Neose is developing proprietary protein drugs that are improved versions of currently marketed therapeutics with proven efficacy. These second generation proteins are expected to offer significant advantages, such as less frequent dosing and improved safety and efficacy. In addition to developing its own products or co-developing products with others, Neose is entering into strategic partnerships for the inclusion of its technologies into products being developed by other biotechnology and pharmaceutical companies.

MacroGenics is a privately funded company focused on the development, manufacture and commercialization of biotechnology products including immunotherapeutics for cancer, infectious diseases, and autoimmune disorders. MacroGenics' core platform involves antibody receptor-related technologies which are employed to improve the ways cytotoxic antibodies mediate cell killing for the treatment of cancers and to prevent autoantibodies from triggering disease in autoimmunity.

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MacroGenics Inc.
Michael Richman
Exec. Vice President and Chief Operating Officer
(301) 251-5172

For more information, please visit www.macrogenics.com.

"Safe Harbor" Statement under the Private Securities Litigation Reform Act of 1995: Statements in this press release regarding our business that are not historical facts are "forward-looking statements" that involve risks and uncertainties. For a discussion of these risks and uncertainties, any of which could cause our actual results to differ from those contained in the forward-looking statement, see the section of Neose's Annual Report on Form 10-K for the year ended December 31, 2003, entitled "Factors Affecting the Company's Prospects" and

discussions of potential risks and uncertainties in Neose's subsequent filings with the SEC.

SOURCE Neose Technologies, Inc.

Neose and Novo Nordisk Sign Research and Development Collaboration Agreement

HORSHAM, Pa., Oct. 1 /PRNewswire-FirstCall/ --
Neose Technologies, Inc. (Nasdaq: NTEC) today announced that it has entered into a research and development collaboration agreement with Novo Nordisk A/S (NYSE: NVO) for the use of Neose's GlycoAdvance™ technology to make clinically significant improvements to a Novo Nordisk therapeutic protein.

We believe Neose's GlycoAdvance technology may make important improvements to marketed therapeutic proteins in the Novo Nordisk pipeline. As a global leader in the development and commercialization of biological products, Novo Nordisk is an ideal partner for GlycoAdvance. We are excited by the potential opportunities to add value to their products through this collaboration, said C. Boyd Clarke, Neose president and chief executive officer.

Novo Nordisk is a focused healthcare company and the world leader in diabetes care. In addition, Novo Nordisk has a leading position within areas such as haemostasis management, growth hormone therapy and hormone replacement therapy. Novo Nordisk manufactures and markets pharmaceutical products and services that make a significant difference to patients, the medical profession and society. With headquarters in Denmark, Novo Nordisk employs approximately 17,900 people in 68 countries and markets its products in 179 countries. For further company information visit www.novonordisk.com.

Neose develops proprietary technologies for using enzymes to manufacture complex carbohydrates. Neose is using its broad technology base to develop novel and improved products for itself and its partners, primarily focusing on protein therapeutics. Neose markets its technology for improving protein drugs under the name GlycoAdvance™. We use GlycoAdvance™ to modify the human carbohydrate structures on therapeutic glycoproteins. We are also developing our technology to create novel glycosylation patterns, and to link other molecules, such as polyethylene glycol, to glycoproteins. The application of this technology to proteins potentially results in improved clinical activity and pharmacokinetic profile, enhanced drug development flexibility, stronger and additional patent claims, and yield improvements. We are exploring the use of our technology to enable the development of carbohydrate-based therapeutics, and the development of novel carbohydrate food and nutritional ingredients.

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Sr. Vice President and Chief Financial Officer

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For more information, please visit www.neose.com.

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SOURCE Neose Technologies, Inc.

-0- 10/01/2002

/CONTACT: Robert I. Kriebel, Sr. Vice President and Chief Financial Officer, or Barbara Krauter, Investor Relations Associate, both of Neose Technologies, +1-215-315-9000/

/Web site: http://www.novonordisk.com /

/Web site: http://www.neose.com /

(NTEC NVO)

CO: Neose Technologies, Inc.; Novo Nordisk A/S
ST: Pennsylvania, Denmark
IN: MTC BIO HEA
SU: JVN CON

FB
-- PHTU058 --
4515 10/01/2002 16:01 EDT http://www.prnewswire.com

Neose Signs R&D and Licensing Agreement with Wyeth-Ayerst

First Commercial Use of GlycoAdvance -- Novel Protein Glycosylation Technology

Horsham, PA, December 19, 2001 -- Neose Technologies, Inc. (NasdaqNM: NTEC) today announced that it has entered into a research, development and license agreement with Wyeth-Ayerst Laboratories, the pharmaceutical division of American Home Products Corporation (NYSE: AHP), for the use of Neose's GlycoAdvance™ technology to develop an improved production system for Wyeth's biopharmaceutical compound, rPSGL-Ig (P-selectin glycoprotein ligand). rPSGL-Ig is a P-selectin antagonist that is being developed to treat inflammation and thrombosis associated with acute coronary syndrome and reperfusion injury. It is currently being evaluated in Phase II clinical trials in patients being treated for heart attack. Wyeth is evaluating the use of GlycoAdvance in the production of rPSGL-Ig for Phase III clinical trials and commercial launch.

Neose will develop processes for the commercial-scale manufacture of proprietary enzymes and sugar nucleotides to be used in the production of rPSGL-Ig, and will license GlycoAdvance to Wyeth for commercial production of the drug. During commercial production of Wyeth's current rPSGL-Ig, Neose will receive ongoing payments tied to yield improvements achieved using GlycoAdvance in the production of rPSGL-Ig. In addition, Wyeth has the option to use GlycoAdvance to develop a next generation rPSGL-Ig, in which case Neose would receive royalties on product sales.

Under the agreement, Neose will receive license, research, and milestone payments that would total up to $17 million if all milestones are met. In addition to ongoing product payments, Neose and Wyeth will also enter into a supply agreement for the long-term supply of GlycoAdvance process reagents.

"We welcome Wyeth as our first commercial partner for GlycoAdvance and look forward to contributing to the success of rPSGL-Ig," says Stephen Roth, Ph.D., Neose's Chairman and CEO. "Wyeth and Neose have worked together extensively to show that GlycoAdvance can be applied to a drug in late stage clinical development. We are particularly excited to be working with Wyeth, given their significant investment in biopharmaceutical drug development, and their commitment to being a world leader in biologics manufacturing."

"GlycoAdvance gives us an important competitive advantage that complements our substantial and growing capital investment in manufacturing capacity," says L. Patrick Gage, Ph.D., President, Wyeth-Ayerst Research. "Using GlycoAdvance with rPSGL-Ig will help us launch the drug with manufacturing capacity in place to supply the projected needs of our initial indication, while giving us the flexibility to supply additional indications as they are developed."

Background on GlycoAdvance

There are more than 360 biotechnology drugs in development for more than 200 diseases. Many of these drugs are glycoproteins - proteins and antibodies that include complex carbohydrates, or sugar chains, as an integral part of their structure. In 2000, worldwide sales of protein and antibody drugs were about $20 billion. By 2010 worldwide sales are expected to exceed $90 billion. Many of these drugs will be glycoproteins and may be appropriate candidates for GlycoAdvance.

GlycoAdvance is Neose's proprietary enzymatic technology for completing the carbohydrate chains on glycoproteins after they have been produced in a biological expression system such as Chinese hamster ovary (CHO) cells. GlycoAdvance uses a class of enzymes, glycosyltransferases, to add individual sugar units onto the carbohydrate structures on glycoproteins. GlycoAdvance can be used with glycoprotein therapeutics, including fusion proteins and monoclonal antibodies, to extend half-life, increase effectiveness and improve manufacturing efficiency.

Glycoproteins contain complex carbohydrate structures attached to the protein portion of the molecule. These carbohydrates are integral to the structure and function of a glycoprotein and help determine how long the drug stays active in the body. Incomplete carbohydrate structures can result in the drug being cleared from the body too quickly, or may result in the drug being less effective. This means that a greater amount of the drug may be required to achieve the intended effect.

Achieving and maintaining the proper carbohydrate structures on glycoproteins is a major challenge in biotechnology manufacturing. Recombinant therapeutic glycoproteins are produced in living cells, usually CHO cells. The use of cell systems to produce glycoproteins requires balancing the cells' ability to produce protein with their ability to put on the required carbohydrates. As the cells' protein output increases, they do not maintain

the proper level of carbohydrates. This often results in low yields of usable product that adds to the cost and complexity of producing these drugs. These low yields are a significant contributor to the critical worldwide shortage of biologics manufacturing capacity.

Background on rPSGL-Ig

Wyeth's rPSGL-Ig is a recombinant version of the human PSGL-1 glycoprotein, linked to the Fc portion of a human antibody. PSGL-1 glycoprotein extends from the surface of white blood cells, or leukocytes, and helps the cells bind to the blood vessel wall in a process known as cell adhesion. PSGL-1 plays a critical role in the migration of these cells from the bloodstream to the site of tissue damage. This is an essential process in helping the body heal itself after an injury. However, in some instances, it can be harmful. Immediately following a heart attack, the leukocytes that attach to the damaged blood vessels exacerbate local inflammation that causes additional tissue damage.

rPSGL-Ig protects the site of tissue damage by preventing leukocytes and platelets from adhering and causing inappropriate inflammation and/or thrombosis. rPSGL-Ig is in Phase II clinical trials evaluating its ability to help accelerate clot dissolution and prevent reperfusion injury following a heart attack. rPSGL-Ig may also have use in solid organ transplantation and arterial vascular diseases including stroke.

About Neose Technologies

Neose develops proprietary technologies for the synthesis and manufacture of complex carbohydrates. The company uses its broad technology platform in the following programs: GlycoAdvance for correcting incomplete or incorrect glycosylation encountered in the manufacture of recombinant glycoproteins; GlycoTherapeutics to develop and produce novel carbohydrate-based therapeutics; and GlycoActives to develop and produce novel carbohydrate-based food ingredients.

Conference Call/Webcast

A conference call and webcast will be held for the investment community on Thursday, December 20, 2001, at 8:30 a.m. EST. The dial-in number for domestic callers is 800-967-7140. The dial-in number for international callers is 719-457-2629. A replay of the call will be available for 7 days beginning approximately four hours after the call's conclusion. The replay number for domestic callers is 888-203-1112 using the passcode 421599. The replay number for international callers is 719-457-0820, also using the passcode 421599. Live audio of the conference call will be simultaneously broadcast over the Internet through World Investor Link's Vcall website, located at www.vcall.com. To listen to the live call, please go to the web site at least fifteen minutes early to register, download, and install any necessary audio software. For those who cannot listen to the live broadcast, a replay will be available shortly after the call.

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"Safe Harbor" Statement under the Private Securities Litigation Reform Act of 1995: Statements in this press release that are not historical facts are "forward-looking statements" that involve risks and uncertainties. Among the risks are that the development of rPSGL-Ig using GlycoAdvance may not succeed, or rPSGL-Ig may not receive regulatory approval or be commercialized successfully. For a more detailed discussion of these risks and uncertainties, any of which could cause the Company's actual results to differ from those contained in any forward-looking statement, see the "Risk Factors" section of Item 1 of the Company's Annual Report on Form 10-K for the year ended December 31, 2000.

Neose Technologies, Inc.

Neose Informed of Wyeth's Intention to Discontinue Development of rPSGL-Ig for Myocardial Infarction

Decision Unrelated to Neose's GlycoAdvance™

HORSHAM, PA, May 9, 2002 -- Neose Technologies (NasdaqNM: NTEC) announced that it has been informed today by Wyeth Pharmaceuticals (NYSE: WYE) that Wyeth does not intend to continue clinical development of their compound, rPSGL-Ig, for myocardial infarction due to disappointing results in Phase II clinical trials. This decision was unrelated to the performance of Neose's GlycoAdvance technology. Although it is possible that development of rPSGL-Ig may continue for other indications, the timing or likelihood of continued development is not known.

Boyd Clarke, president and CEO of Neose Technologies, said, "We knew this compound was in Phase II clinical trials, and was subject to the normal risks and uncertainty associated with clinical drug development. Although our technology was working as planned, we are disappointed by the news from Wyeth. We hope to continue working with Wyeth to use GlycoAdvance in their therapeutic protein development programs."

Neose develops proprietary technologies for the synthesis and manufacture of complex carbohydrates. The company uses its broad technology platform in the following programs: GlycoAdvance products and services for correcting incomplete or incorrect glycosylation encountered in the manufacture of recombinant glycoproteins; GlycoTherapeutics™ to develop and produce novel carbohydrate-based therapeutics; and GlycoActives™ to develop novel carbohydrate-based food and nutritional ingredients.

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Neose Technologies, Inc. and Monsanto Protein Technologies Sign Research Agreement

HORSHAM, Pa., and ST. LOUIS, Nov. 25 /PRNewswire-FirstCall/ -- Neose Technologies, Inc. (Nasdaq: NTEC) and Monsanto Protein Technologies, a unit of Monsanto Company (NYSE: MON), today announced that they have entered into a research agreement to investigate the use of Neose's GlycoAdvance(TM) technology to enhance the glycosylation of therapeutic monoclonal antibodies produced in plants.

Today, the majority of therapeutic monoclonal antibodies are produced by mammalian cell culture. The glycosylation patterns of plant-produced monoclonal antibodies differ significantly from monoclonal antibodies produced by mammalian cell culture. Monoclonal antibodies produced in plants have incomplete glycosylation patterns, resulting in the inability to activate the complement system and other types of critical effector function. This research will combine Monsanto Protein Technologies' expertise in transgenic plant production of monoclonal antibodies with Neose's expertise in glycosylation. This is expected to enhance the ability of plant-produced monoclonal antibodies to initiate complement activation.

"Monsanto believes this science promises tremendous benefits and will someday provide greater access to life-saving therapeutic drugs, thus providing more options for patients and doctors," said Cheryl Morley, President of Monsanto's Animal Ag and Protein Technologies Group. "We are excited about working with Neose and the GlycoAdvance technology."

"GlycoAdvance has the potential to make an important contribution to the glycosylation, and therefore the therapeutic usefulness of plant-produced proteins. We are delighted to be working with Monsanto, a leader in the development of plant-based systems," said C. Boyd Clarke, Neose president and chief executive officer.

Monsanto Protein Technologies, a unit of Monsanto, is focused on contract manufacturing of therapeutic proteins at very large scale derived from plant biotechnology. Monsanto is recognized as a leader in plant biotechnology and recombinant protein technology. By leveraging this breadth of experiences ranging from genomics and seed breeding, to large scale sterile protein manufacturing, Monsanto Protein Technologies can deliver a cost-effective process for producing therapeutic proteins at very large scale.

Neose develops proprietary technologies for using enzymes to manufacture complex carbohydrates. Neose is using its broad technology base to develop novel and improved products for itself and its partners, primarily focusing on protein therapeutics. Neose markets its technology for improving protein drugs under the name GlycoAdvance. GlycoAdvance is used to modify the human carbohydrate structures on therapeutic glycoproteins. Neose is also developing its technology to create novel glycosylation patterns, and to link other molecules, such as polyethylene glycol, to glycoproteins. The application of this technology to proteins potentially results in improved clinical activity and pharmacokinetic profile, enhanced drug development flexibility, stronger and additional patent claims, and yield improvements.

For more information pertaining to Neose Technologies, Inc., please visit http://www.neose.com.

For more information pertaining to Monsanto Protein Technologies, please visit http://www.mpt.monsanto.com.

Cautionary Statements Regarding Forward-Looking Information of

Neose Technologies, Inc.:

Statements in this press release regarding our business that are not historical facts are "forward-looking statements" that involve risks and uncertainties. For a discussion of these risks and uncertainties, any of which could cause our actual results to differ from those contained in the forward-looking statement, see the "Risk Factor" section of Item 1 of our Annual Report on Form 10-K for the year ended December 31, 2001.
Cautionary Statements Regarding Forward-Looking Information of Monsanto Company and Monsanto Protein Technologies (a unit of Monsanto Company):

Certain statements contained in this release, such as statements concerning the anticipated financial results, current and future product performance, regulatory approvals, currency impact, business and financial plans and other non-historical facts pertaining to Monsanto Company or Monsanto Protein Technologies, Inc. are "forward-looking statements." These statements are based on current expectations and currently available information. However, since these statements are based on factors that involve risks and uncertainties, the actual performance and results of Monsanto Company or Monsanto Protein Technologies, Inc. may differ materially from those described or implied by such forward-looking statements. Factors that could cause or contribute to such

differences include, among others: the success of the research and development activities of Monsanto Company and Monsanto Protein Technologies, Inc. and the speed with which regulatory authorizations and product launches may be achieved; the ability of Monsanto Company or Monsanto Protein Technologies, Inc. to successfully market new and existing products in new and existing domestic and international markets; the ability of Monsanto Company or Monsanto Protein Technologies, Inc. to achieve and maintain protection for its respective intellectual property; the exposure of Monsanto Company or Monsanto Protein Technologies, Inc. to lawsuits and other liabilities and contingencies, including those related to intellectual property, product liability, regulatory compliance, environmental contamination and antitrust; and other risks and factors detailed in the Monsanto Company's filings with the U.S. Securities and Exchange Commission. Undue reliance should not be placed on these forward-looking statements, which are current only as of the date of this release. Monsanto Company and Monsanto Protein Technologies, Inc. disclaim any current intention to revise or update any forward-looking statements or any of the factors that may affect actual results, whether as a result of new information, future events or otherwise.

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